The structure, expression and function of the *PHANTASTICA* gene in *Antirrhinum* organ development

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Abstract

The mutant phenotype of the *PHANTASTICA (PHAN)* gene had indicated that *PHAN* was required for dorsoventrality of leaves, bracts and petal lobes in *Antirrhinum majus*. Here it was shown that at low temperature, *phan* mutants carrying a likely null allele also stopped organ initiation and growth, and the shoot apical meristem arrested. These effects were reversed when plants were returned to a higher temperature. This indicated that *PHAN* is also required redundantly for earlier elaboration of the proximodistal axis of all organs and for meristematic function.

PHAN was shown to encode a novel MYB-related transcription factor. *In situ* hybridisation revealed that *PHAN* mRNA was first expressed in leaf initials before they became morphologically distinct from the meristem. Comparisons of the expression patterns of *PHAN* and floral homeotic genes revealed that *PHAN* is expressed in floral organ initials at, or before, the time that their identities are specified within the meristem.

The early and specific expression pattern of *PHAN*, its requirement for elaboration of the two axes unique to lateral organs and the regulatory activity of its product suggest that it specifies the identity of lateral organ initials as distinct from the meristem and stem. The requirement for *PHAN* in maintenance of the shoot apical meristem appears to be non cell-autonomous and therefore to involve *PHAN*-dependent signalling from organ initials.

To characterise the relationship between *PHAN* and other genes involved in meristem function, the expression pattern of an *Antirrhinum* homologue of the *Arabidopsis SHOOTMERISTEMLESS* (*STM*) gene, *AmSTM*, was determined. This showed that *AmSTM* was expressed in a domain complementary to that of *PHAN*. Evidence suggesting that *PHAN* is required for expression of *AmSTM* was obtained.

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phantastica (*phan*) is a recessive mutation affecting the shape of leaves, bracts and petal lobes (Waites and Hudson, 1995). The most extreme mutant phenotype is a drastically reduced leaf which is needle-like in appearance and is radially symmetrical in transverse section. Needle-like leaves consist entirely of ventral cell types arranged in concentric cylinders, lacking dorsoventral asymmetry. It was observed that lateral expansion and cells characteristic of wild type lamina were absent. The epidermis of these leaves is similar to that of the ventral midrib of a wild type leaf, although it displays some wild type characters, such as the cells being shorter and the presence of stomata over the leaf surface.

The cotyledons and the first three pairs of leaves of *phan* mutants are broader and contain more cells in transverse section than in wild type plants. These leaves also have ectopic patches of ventral cell types on the dorsal surface. At the boundary of these regions and the dorsal epidermis, ectopic laminal ridges are formed. Needle-like leaves are formed late in vegetative development. Leaves at intermediate nodes are usually narrower than wild type, or are mosaics of needle-like ventral tissue and laminal tissue at proximal or distal positions. Narrow leaves have fewer cells in transverse section and their midrib arises at a more dorsal position. Mosaic leaves have both needle-like and laminal tissue in proximal or distal positions. At the boundary of these two tissue types, an ectopic lamina is formed.

All *phan* alleles show the same vegetative phenotype, but each has a characteristic floral phenotype where the petal lobes show a loss of dorsoventrality, similarly to leaves. The dorsal surface of the petal lobes is characterised by conical shaped cells while the ventral cells are smooth with hairs (Noda *et al.*, 1994). All other floral organs appear wild type. The corolla tube of the flower is unaffected and can be thought of as being homologous to the petioles of leaves which are unaffected in *phan* plants.

Waites and Hudson (1995) put forward a model to interpret the *phan* mutant phenotype. They suggested that *PHAN* was required for a dorsalising function (DF) which determines dorsal cell identity and acts in the dorsal region of the leaf primordia. The juxtapositioning of the ventral and dorsal domains leads to lateral proliferation to form the lamina. In needle-like leaves, there has been a total loss of DF so that there are no dorsal cell types and no lateral proliferation. Narrow leaves can be explained by the shifting of the domain of DF to a more dorsal position so that the lamina is formed at a more dorsal position to leave a greater proportion of the primordia with ventral identity. Since the primordium is widest at its midpoint, shifting the boundary to a more dorsal position where there are fewer laminal cells, will result in a narrower leaf. Mosaic leaves can be accounted for by the shifting of the domain of DF to more distal or more proximal positions in the leaf primordia, such that lamina tissue is formed in a novel axis at the boundary of both domains. Broader early leaves have lost DF in patches so that ectopic proliferation occurs at the boundary of these patches and the surrounding dorsal cells, which gives an increased number of cells.

This model is also applicable to petal lobes and bracts to reinforce the view that they are homologous structures. The range of leaf phenotypes found on the same plant indicates that the is a different requirement for *PHAN* in DF as the plant matures such that younger leaves have a greater requirement for *PHAN*. Similarly bracts and petal lobes have a greater requirement for *PHAN* than early leaves. It is likely that other factors determine the dorsoventrality of other organs.

In order to characterise the definition of dorsoventrality, the *PHAN* gene had been isolated by Richard Waites (Waites *et al.*, 1998).

Therefore the aim of this work is to relate the structure and expression pattern of *PHAN* to its function in organ development. It is shown that *PHAN* has the potential to encode a MYB-related transcription factor and that the timing and pattern of *PHAN* mRNA expression coincides with the determination of lateral organ identity.

Not only is *PHAN* shown to function in dorsoventrality, but also in all aspects of specifying organ fate within the meristem.

Development of the Aerial Parts of *Antirrhinum majus*

As with most higher plants, the above ground part of *Antirrhinum* is generated from the vegetative shoot apical meristems (SAM), which is formed in the embryo. Following germination of the seed, the vegetative meristem produces a pair of leaves at each node, at 180° to each other in a decussate phyllotaxy (Figure 1). The leaves of *Antirrhinum* are typical of many dicots, having a simple ovate shape with an entire margin. The shoot branches by the outgrowth of axillary meristems (in the axil of the leaf and stem) to form new lateral shoots. The single repeating unit (phytomer) of the vegetative phase therefore consists of internode, node, leaf, and axillary bud.

After a period of vegetative growth, reproductive growth is initiated and the vegetative meristem becomes an inflorescence meristem (Figure 1). This produces smaller leaf-like organs called bracts which are formed singly in a spiral phyllotaxy and are separated by shorter internodes. This phase can also be regarded as late vegetative, such that the vegetative meristem produces leaves, then bracts, after which the inflorescence meristem produces floral meristems (Hempel and Feldman, 1994). Many plants show changes in vegetative characters like leaf shape and the density and/ or distribution of trichomes, to differentiate between juvenile and mature leaves (e.g. Martinez-Zapater *et al.*, 1995; Lawson and Poethig, 1995).

Once the inflorescence stage is established, a floral meristem is then initiated in the axil of each bract (Figure 1) The floral meristem goes on to produce four concentric whorls of organs separated by very short internodes. Floral organ primordia initiate sequentially on the flanks of the floral meristem. Five sepals first, followed by five petals and almost simultaneously, four stamens (one stamen primordium is arrested in development) and finally, two united carpels (Awasthi *et al.*, 1984). The floral meristem is determinate in that it forms a fixed number of whorls of organs, while both the vegetative and inflorescence meristems are indeterminate, having the ability to carry on producing organs indefinitely.

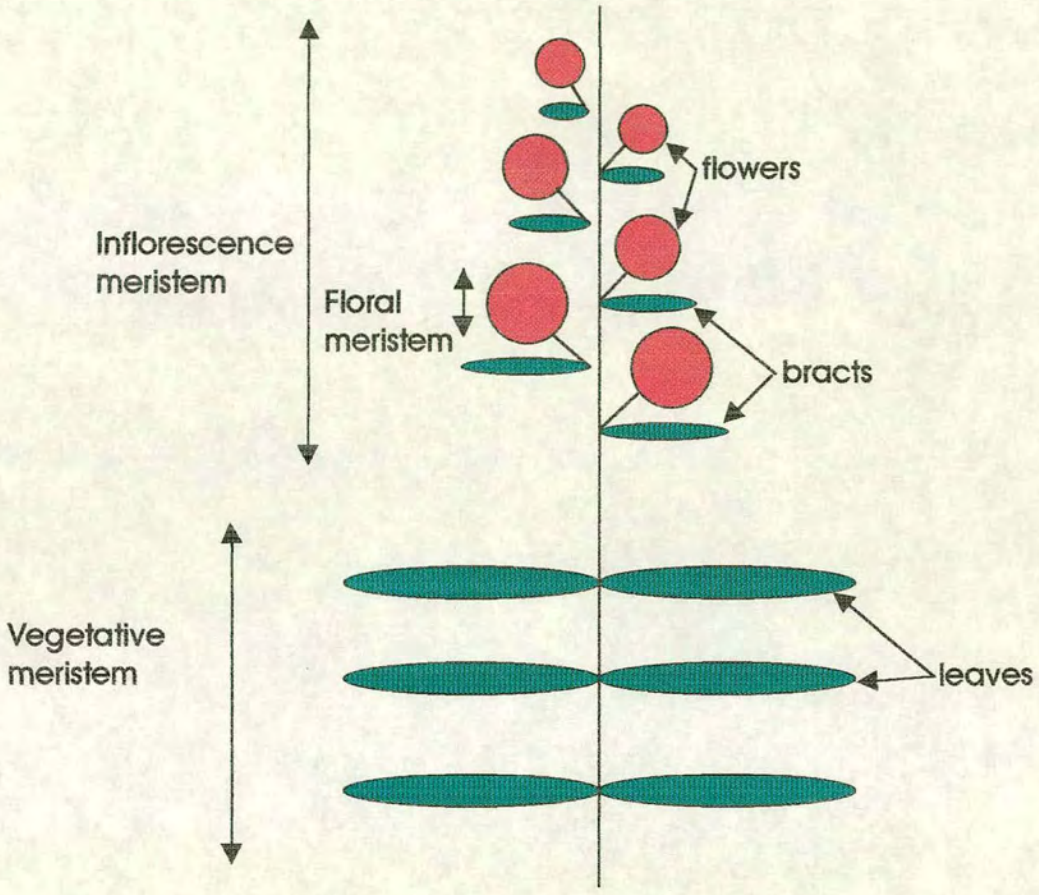


Figure 1. Schematic diagram to show the aerial development of *Antirrhinum*. The vegetative meristem produces leaves, while the inflorescence meristem produces bracts and floral meristems.

Antirrhinum flowers are symmetrical about their vertical axis (zygomorphic). Organs within the flower are referred to as dorsal or ventral, depending on their relative positions, where bract is ventral and stem is dorsal. The ventral two sepals are alternate to the bract. The five petals which form alternately with the sepals, unite to form the corolla tube and then separate to form five petal lobes. The upper two lobes are distinct from the lower three lobes which are united to form a hinge region. In the next whorl, five stamens are initiated alternative to the petals. One stamen primordium fails to develop so that there are only four stamens in the mature flower. The last whorl consists of two carpels which united give a gynoecium having a bilocular ovary.

The youngest floral meristems are at the top of the inflorescence apex, with progressively later stages below. Carpenter *et al.* (1995) divide floral meristem development into 6 stages (Figure 2). During the bract tongue stage (nodes 0-4) the bract primordium appears as a small mound on the periphery of the inflorescence apex and goes on to produce a tongue shaped primordium separated from the inflorescence meristem by a crease. The eye stage (nodes 4-8) describes the eye shaped floral meristem which is visible in the axil of the bract primordium, which is by now hooded in shape. By the end of this stage the floral meristem has taken on a more elliptical appearance. By the loaf stage (nodes 8-10) the floral meristem is more rectangular in shape and has risen up like a loaf of bread. At the pentagon stage (nodes 10-12) the floral meristem takes on a pentagonal shape. The corners of the meristem correspond to sepal initials. By the end of this stage the sepal primordia are visible as bulges from the meristem. The floritypic stage (nodes 12-14) sees the sepals clearly separated from the meristem by a crease. This stage is common to many species and is when several key homeotic genes are activated (Bradley *et al.*, 1993). By the petal mound stage (nodes 15-18), the sepals are arched over the meristem, the petal primordia can be seen as small mounds at the vertices of the inner pentagon of the floral meristem. The stamen primordia are visible as undulations on the surface of the meristem. By node 18, the petal primordia are tongue like and the four stamens are rounded domes, with the fifth stamen primordium being retarded in its development.

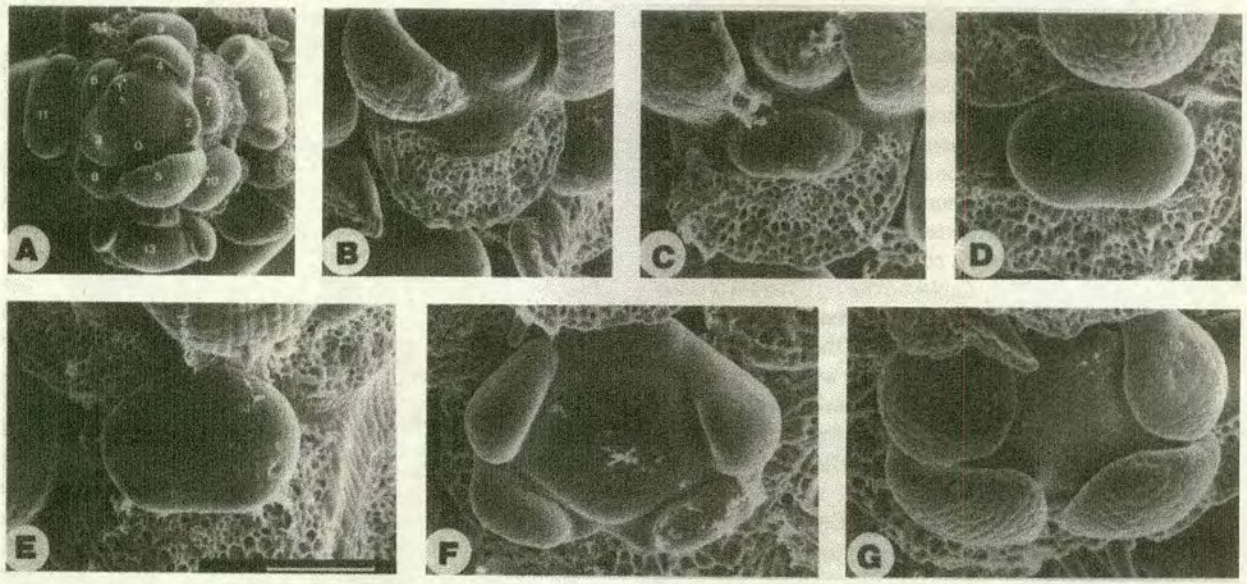


Figure 2. Scanning electron microscopy of different stages of *Antirrhinum* floral development (Carpenter *et al.*, 1995). (A) Inflorescence apex with nodes numbered sequentially. (B) Stage 1, eye. (C) Early stage 2, loaf. (D) Late stage 2, loaf. (E) Stage 3, pentagon. (F) Stage 4, floritypic. (G) Stage 5, petal mound. Bar in (E) = 100 μm.

The development of *Antirrhinum* involves a transition from vegetative meristem, to inflorescence and finally to floral meristem. This transition is accompanied by a change in phyllotaxy, internode length, a switch from indeterminate to determinate growth, and the identity of organs formed. What is the genetic basis for these changes? Analysis of homeotic mutants provides some clues. In *Antirrhinum*, there are two main classes of homeotic genes. Firstly, genes controlling meristem identity, e.g., *FLORICAULA* (*FLO*) (Coen *et al.*, 1990) and *SQUAMOSA* (*SQUA*) (Huijser *et al.*, 1992), and secondly, genes controlling organ identity, e.g., *PLENA* (*PLE*) (Bradley *et al.*, 1993), *DEFICIENS* (*DEF*) (Sommer *et al.*, 1990; Schwarz-Sommer *et al.*, 1992) and *GLOBOSA* (*GLO*) (Trobner *et al.*, 1992). A further class including *FIMBRIATA* (*FIM*) (Simon *et al.*, 1994) has been shown to mediate between these two.

flo mutants have wild type vegetative meristems which switch to inflorescence meristems as in wild type (Coen *et al.*, 1990). Bracts are formed from the flanks of the inflorescence meristem, but then, instead of initiating a floral meristem in the axil of the bract, an indeterminate shoot bearing further bracts is produced. This shoot has two bracts at its base followed by single bracts at each node in a spiral arrangement and further shoots can be initiated in the axils of these bracts. This is indicative of indeterminate inflorescence meristems growing in the place of determinate floral meristems. Therefore, the wild type *FLO* is required for the transition from inflorescence to floral meristem identity in axillary positions. *In situ* hybridisation showed that as expected, *FLO* mRNA is expressed in very young inflorescences. The earliest stage of *FLO* expression can be seen to mark floral meristems at the bract tongue stage before they are visible, and in bract 0, before it emerges from the flanks of the inflorescence meristem. Expression is subsequently observed in sepal, petal and carpel primordia which correspond to whorls 1, 2 and 4 respectively, with expression being stronger on the dorsal sides of primordia and lasting for about 5-10 nodes. This suggests that *FLO* may not only act to switch from inflorescence to floral meristem identity, but may also be involved in directing gene expression in later floral meristems. The expression of *FLO* in specific primordia may

be necessary to set up patterns of gene expression that regulate their development. *FLO* can be thought of as being a primary regulator since it is expressed transiently, in contrast to genes affecting whorl identity, like *DEFICIENS* (*DEF*) which is expressed throughout petal development (Carpenter and Coen., 1990) at constant levels (Sommer *et al.*, 1990). *SQUA* (Huijser *et al.*, 1992) has a similar mutant phenotype to that of *FLO* except that an abnormal flower is eventually produced by the axillary shoots. As with *FLO*, *SQUA* is expressed in eye stage floral meristems, but only weakly in bract primordia (Carpenter *et al.*, 1995; Huijser *et al.*, 1992). *FLO* expression in *squa* mutants was much as in wild type. In *flo* mutants, *SQUA* was expressed at near wild type levels, but there was also some ectopic expression in the inflorescence apex. These results suggest that *FLO* and *SQUA* are expressed independently. Similarly, *FLO* mRNA expression in *flo* mutants was reduced and ectopic expression also occurred in the inflorescence apex. This suggested that a minimal level of *FLO* transcript is required to autoregulate its own expression up to a critical level, presumably facilitating a discrete switch from inflorescence to floral meristem identity (Carpenter *et al.*, 1995). *FLO* then goes on to act non-cell-autonomously to down regulate *FLO* and *SQUA* expression at the apex. So, although *FLO* and *SQUA* are activated independently, they interact to promote floral development (Figure 3). Double mutants were made to investigate the interaction of *FLO* and *SQUA* (Carpenter *et al.*, 1995). The *squa* phenotype is more extreme when the levels of *FLO* transcript are reduced in *flo*/*Flo*$^+$ heterozygotes, while *Flo*$^+$/*Flo*$^+$ homozygotes produced the normal *squa* phenotype. Thus, Carpenter *et al.*, (1995) concluded that *FLO* can be regarded as epistatic to *SQUA*, although double mutants having a weak *squa* allele were not analysed. A class of genes that may be regulated by *FLO* and *SQUA* are the homeotic genes regulating whorl identity.

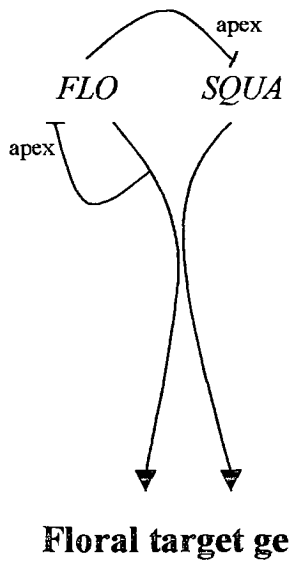


Figure 3. Flow diagram to show the interaction of *FLO* and *SQUA* in the regulation of floral organ identity. *FLO* down regulates it's own expression and the expression of *SQUA* at the apex.

Mutations in organ identity genes affect the identity of organs produced, usually in two adjacent whorls. The mutations have been classified according to the loss of three homeotic functions, *a*, *b*, or *c*. *a* function mutants have carpels instead of sepals and stamens instead of petals. *b* function mutants have sepals in place of petals and carpels in place of stamens. *c* function mutants have multiple whorls of petals or sepals instead of stamens and carpels. Carpenter and Coen (1990) proposed that these three homeotic functions act in a combinatorial manner where whorl 1 expresses *a*, whorl 2 expresses *a* and *b*, whorl 3 expresses *b* and *c*, with whorl 4 expressing just *c* (Figure 4). *def* and *globosa* (*glo*) mutants are *b* function defective, *ple* mutants lack *c* function, but no loss of *a* function mutants are known for *Antirrhinum*. *DEF* (Sommer *et al.*, 1990), *GLO* (Trobner *et al.*, 1992) and *PLE* (Bradley *et* al., 1993) have been isolated and their expression patterns characterised. *DEF* and *GLO* are expressed in whorls 2 and 3, while *PLE* is expressed in whorls 3 and 4. DEF and GLO proteins have been shown to form heterodimers to regulate the activity of their target genes, and also to positively autoregulate their own transcription (Schwarz-Sommer *et al.*, 1992; Trobner *et* al., 1992; Zachgo *et* al., 1995). PLE and SQUA have also been shown to interact (Davies *et al.*, 1996). The expression patterns of both genes overlap during floral meristem development and could result in the downregulation of *SQUA* expression in the third whorl, or it could be part of the pathway which results in determinacy, explaining the lack of determinacy seen in flowers produced by a *squa* mutants (Davies *et al.*, 1996; Huijser *et al.*, 1992)). The interaction between PLE and SQUA suggests a link between the establishment of a floral meristem and the determination of organ identity (Davies *et al.*, 1996).

FIM mutations lead to the partial homeotic transformation of floral organs, affecting whorls two, three and four, and a reduction in the determinacy of the floral meristem. Molecular and genetic epistasis experiments suggest that *FIM* expression is dependent on *FLO* in floral meristems and required for the correct expression of *DEF* and *PLE*, indicating that it mediates between meristem identity genes and organ identity genes (Simon *et al.*, 1994). Unlike *DEF* and *PLE*, *FIM* is expressed

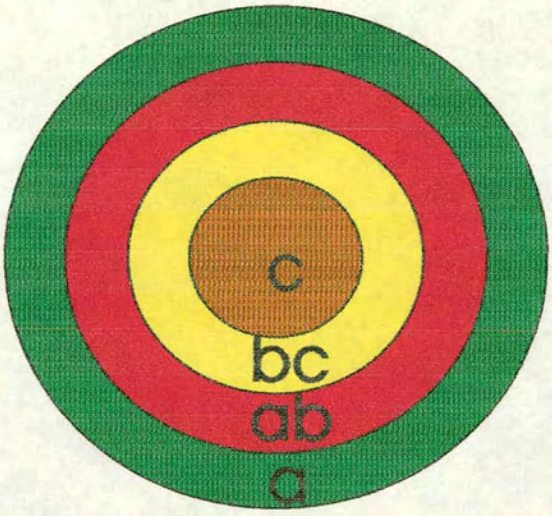


Figure 4. *abc* functions determined by organ identity genes. In whorl 1, *a* specifies sepals (green); in whorl 2, *a* and *b* specify petals (red); in whorl 3, *b* and *c* specify stamens (yellow); in whorl 4, *c* specifies carpels (brown).

transiently in early floral meristems (at the ventral side of the central dome at first and then more centrally) and becomes restricted to a ring at the base of the whorl one and whorl two primordia. This pattern of expression is complementary to the pattern of *FLO* expression, where *FLO* is expressed in sepals and the central parts of petal primordia. This can be explained by the two genes negatively regulating each other to define precise domains of expression. Weak ectopic *FIM* expression is seen the main and axillary inflorescence apices of *flo* mutants, indicating that while *FIM* can be expressed independently of *FLO*, *FLO* acts to restrict *FIM* activity in specific regions. Since *flo* null mutants produce no flowers at all, *FLO* is expected to be epistatic to *FIM*. In agreement with this, a weak *flo* allele enhances the *fim* phenotype, indicating that a reduction in *FLO* leads to a reduction in *FIM* activity. The expression patterns of *FIM* and *SQUA* overlap suggesting that they act together. In *squa* mutants, *FIM* is not expressed in the main or axillary inflorescence meristems, but after a delay of approximately six nodes, there is expression of *FIM* in the floral meristems in a pattern similar to wild type, suggesting that *SQUA* is involved in setting the time of *FIM* activation. Analysis of *fim;squa* mutants reveals that while *squa* single mutants usually produce abnormal flowers, the double mutants produce only inflorescences. This suggests that *FIM* and *SQUA* act together to encourage the switch to floral meristem identity. The reduction of one dose of *SQUA* in a *fim* mutant background leads to an extreme *squa* phenotype, while *squa* is fully recessive in a wild type background. Therefore, in *fim* mutants, the move to floral meristem identity appears to be at a *SQUA* dependant stage. *FIM* seems to promote *DEF* and *PLE* expression since in *fim* mutants, expression of *DEF* and *PLE* is reduced and delayed. In wild type, by the floritypic stage, the domain of *PLE* extends out to and stops sharply at the inner boundary of the ring of *FIM* expression. The outer boundary of *DEF* overlaps that of *FIM* expression. The expression patterns of both these organ identity genes is altered in *fim* mutants such that the outer boundaries are shifted towards the centre of the meristem. In early stages of floral development, there is no change in *FIM* expression in *def* and *ple* mutants, with *FIM* forming a ring of expression at the base of whorl one and two primordia. Later in *ple* mutants, expression of *FIM* is also seen at the base of all internal organs. This indicates that *FIM* may function to set the domains of expression of organ identity genes to coincide with the morphological

boundaries that have, or are about to be set, between whorls. Double mutants of *fim*, *def* and *ple* were constructed to show any other functions of *FIM*. The *def;fim* mutant was similar to the *def* single mutant with delayed carpel formation and loss of determinacy, characteristic of a loss of *PLE* activity. The *fim;ple* double mutants were similar to *def;ple* double mutants, but additionally, there was a loss of lateral determinacy, not observed in the *def*;ple mutants. *fim* mutants sometimes initiate inflorescences or flowers in the axils of floral organs, so that in the wild type, *FIM* probably acts with meristem identity genes to establish lateral determinacy.

The genetic interactions that determine floral development in *Antirrhinum* have many parallels in homologous mutations in *Arabidopsis* (Figure 5). A high level of structural and functional homology has been seen between the two species (reviewed by Weigel and Meyerowitz, 1994).

Function	*Antirrhinum*	*Arabidopsis*
Meristem Identity	*FLORICAULA*, *SQUAMOSA*	*LEAFY*, *APETALA1*
a Function		*APETALA2*
b Function	*DEFICIENS*, *GLOBOSA*	*APETALA3*, *PISTILLATA*
c Function	*PLENA*	*AGAMOUS*
Mediating	*FIMBRIATA*	*UNUSUAL FLORAL ORGANS*

Figure 5. Homologous genes in *Antirrhinum* and *Arabidopsis* floral development.

The Shoot Apical Meristem

Introduction

Apical meristems at the growing tips of plants give rise to the majority of the plant body. In general, the shoot apical meristem (SAM) generates the majority of the aerial parts of the plant, while the root apical meristem produces the subterranean parts. The word "meristem" is used to here to describe a highly organised structure that is responsible for organ formation, as opposed to merely actively dividing parts of the plant. Meristems consist of self-renewing populations of stem cells at the apex. Daughter cells which remain at the apex retain stem cell identity, while those passing to the flanks of the meristem assume organ initial identity (e.g. leaves) and internode. After being specified during embryogenesis, the SAM may adopt one of three different fates, vegetative (juvenile and adult), inflorescence and floral. These meristems differ mainly in the identity of organ produced and their positions. All are indeterminate, except for floral meristems, in which the central stem cell populations are lost to carpel primordia which form directly from the apex.

Structure of the SAM

The size and shape of meristems vary greatly, however the internal organisation of most angiosperm SAMs is quite similar. Cytohistological staining and differences in the number and arrangement of cell divisions, have identified three zones within the meristem (reviewed in Steeves and Sussex, 1998), (Figure 6). The central zone (CZ) is the region at the apex of the meristem, and comprises cells that are large, highly vacuolated and relatively slowly dividing. The morphogenetic or peripheral zone (PZ) is the region at the flanks of the meristem. The PZ consists of smaller, more rapidly dividing cells with small vacuoles. Cell divisions at the central zones serve to maintain a steady number of indeterminate cells in the CZ. Cells from the CZ are incorporated into the PZ to form initials which go on to become organ primordia. The lower region of the SAM is termed the rib zone (RZ) which is made up of rapidly dividing cells and contribute to the stem. Below these three zones of the

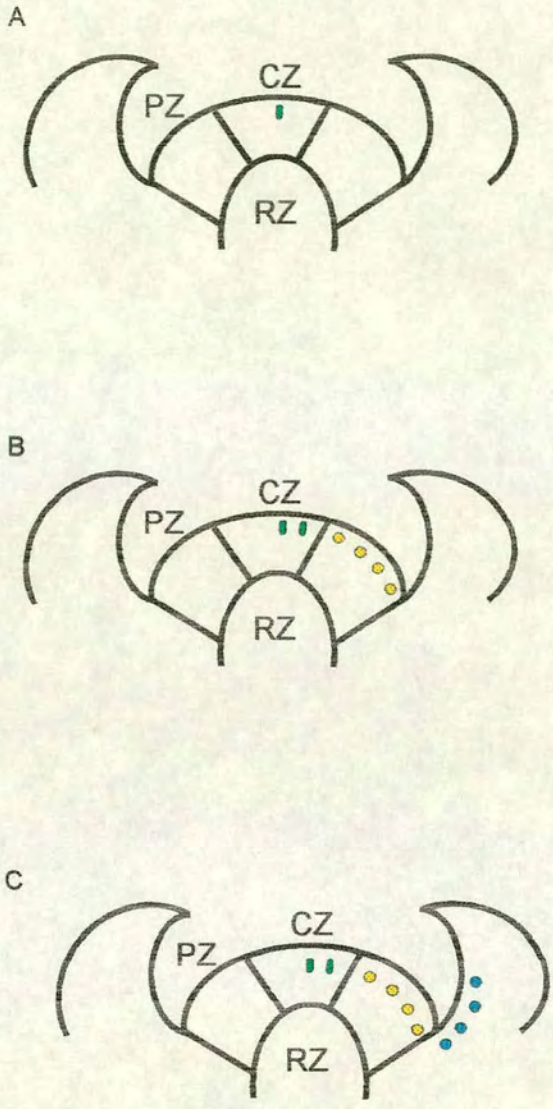


Figure 6. Schematic diagram showing the dynamics of the meristem (modified from Clark, 1997)

(A) A single undiferentiated cell [•] in the CZ from which cells in (B) and (C) are derived.
(B) Progeny cell [•] on the flanks where organ initials are organised.
(C) Cells formerly in the PZ divide as organ primordia grow [•].
CZ = central zone, PZ = peripheral zone and RZ = rib zone.

SAM, is the zone of maturation (ZM) where there is a considerable increase in the width of the shoot and the size of primordia. The meristem is therefore a dynamic structure with cells moving from central regions to the flanking regions.

The meristem is also arranged in cell layers. The outermost layer, L1, generates the epidermis, while the inner layers, L2 and L3 contribute to the inner tissues of leaf and stem. Both L1 and L2 are one cell thick and are together termed the tunica (Hanstein, 1886). Here cell divisions are anticlinal, i.e. they are perpendicular to the surface of the apex, so that cell lineage is maintained (Satina *et al.*, 1940). In contrast, the L3 (corpus) (Schmidt, 1924) comprises of several layers which divide anticlinally and periclinally, parallel to the surface of the apex. The use of periclinal chimeras, where one cell layer is genetically distinct from the others, has shown that the layered organisation of the meristem is maintained, although migration of cells from one layer to another can occur rarely (Dermen, 1953; Stewart and Burk, 1970; Stewart and Dermen, 1979). Sometimes, cells from the L1 or L2 divide periclinally to form new cell walls that are parallel to the surface of the layer. This means that a daughter cell invades an adjacent layer. It was found that this cell now goes on to adopt the fate of the new layer rather than the original layer, indicating that cell fate is primarily determined by position rather than lineage (Dermen, 1953; Stewart and Burk, 1970; Stewart and Dermen, 1979).

Meristem Specific Gene Expression

Numerous genes with meristem specific patterns of expression have been identified (Medford, 1992), some reflecting tunica/corpus and zonation patterns of the meristem, as detected by histological analyses (Fleming *et al.*, 1993; Kelly and Meeks-Wagner, 1995; Lu *et al.*, 1996). Others reveal domains of the meristem that were previously unidentified by structural features (Smith *et al.*, 1992; Souer *et al.*, 1996). However, a relatively small number of genes showing a meristem specific effect have been identified. It is possible that meristem regulatory genes are redundant, or have pleiotropic effects so that their roles in the meristem are masked.

The *KNOTTED1* (*KN1*) gene of maize has proved to be a useful marker for meristem function. *KN1* encodes a homeodomain protein which is expressed at low levels in the leaves of wild type plants (Vollbrecht *et al.*, 1991; Smith *et al.*, 1992). It is expressed strongly in the meristem and in the ground tissue of the unexpanded stem. However, it is not expressed in the predicted position of leaf initials within the meristem. Dominant mutants of *KN1* are characterised by localised regions of extra cell division in all cell layers of the leaf which gives rise to knots on the leaf blade (meristem-like characteristic) (Bryan and Sass, 1941), but is determined cell non-autonomously by *KN1* in a subgroup of cells in the inner layer (Sinha and Hake, 1990). This knotted phenotype was shown to correspond to ectopic expression of the KN1 protein in leaf veins of mutant plants. Therefore, it was hypothesised that *KN1* is expressed in the meristem to maintain these cells in an undifferentiated state, and that loss of *KN1* expression is necessary for the initiation of determinate lateral organs, like leaves (Smith *et al.*, 1992). To further understand the role of *KN1*, loss-of-function mutations were identified (Kerstetter *et al.*, 1997). Plants homozygous for the recessive *kn1* alleles had a reduced level of KN1 protein as well as transcript. The phenotype showed reduced inflorescence branching and proliferating pistils. A small proportion of plants had extra leaf portions in the axils of leaves. Since the SAM does not over produce lateral organs and /or cease to function due to reduced levels of *KN1* expression, it can be assumed that the function of *KN1* in maintaining meristem cells, is at least partially redundant.

The *KN1* like gene, *SHOOTMERISTEMLESS* (*STM*) from *Arabidopsis* (Kerstetter *et al.*, 1994) is required for the formation of the SAM during embryogenesis. The null mutant, *stm*-1 produces normal mature embryos, except for the lack of a SAM (Barton and Poethig, 1993). In wild type plants, it was found that *STM* expression precedes SAM initiation and is localised to the cells that are predicted to form the embryonic SAM (Long *et al.*, 1996). *STM* expression is present in all types of meristems, vegetative, axillary, inflorescence and floral (Long *et al.*, 1996). The phenotype of weaker alleles has suggested that *STM* is also required post-embryonically to maintain meristem function (Clark *et al.*, 1996). In vegetative meristems, there is strong *STM* expression in the meristem, but is absent in leaf

primordia or leaves. Expression is also down regulated in regions of the meristem which are predicted to give rise to leaf primordia (i.e. leaf initials). Similarly, in inflorescence meristems, *STM* is expressed in the meristem, but is absent from domains that will form cauline leaves, secondary inflorescences and floral meristems. Subsequently, *STM* expression is re-established in floral meristems before they initiate floral organs. While *STM* expression is absent in the floral organ primordia, expression is seen in carpels in two ridges of tissue which will form ovules. It is probable that these ridges behave like meristems to generate ovules. *STM* mRNA also accumulates in the vascular tissue of the stem. Plants homozygous for the strong *stm*-1 allele, occasionally produce "rescued" shoots at positions above the junction of cotyledon vascular strands. These shoots initiate leaves very slowly in a manner uncharacteristic of a meristem, suggesting that an organised SAM may not be necessary to form leaves (Barton and Poethig, 1993).

Analysis of weak *stm* alleles (Clark *et al.*, 1996; Endrizzi *et al.*, 1996) showed that while there was no fully active meristem in the postembryonic stages, two or three leaves can be formed and new meristems can be formed in the axils of these leaves. These adventitious meristems terminate after forming a few leaves, revealing a continued requirement for *STM* activity to maintain functional meristems. In the embryonic stages of these weak alleles, there are some cells having meristematic features at the apical pole. This could indicate an embryonic SAM which has not fully developed, or it could be the formation of an adventitious meristem. Therefore, there are two possible roles for the action of *STM* in specifying and maintaining the meristem. Firstly, *STM* could be required to regulate cell division in the CZ, such that in *stm* mutants, there is insufficient replenishment of the CZ. Alternatively, *STM* could act to keep cells in an undifferentiated state so that in *stm* mutants, cells in both the CZ and PZ become differentiated. Thus, in wild type meristems, the loss of *STM* expression is sufficient to specify organ fate.

The *CLAVATA* (*CLV1* and *CLV3*) loci may act to promote the transition to the differentiated state in cells of the CZ. They may also restrict cell division in this zone (Clark *et al.*, 1993, 1995). The two genes *CLV1* and *CLV3* are likely to function in

the same pathway since mutant alleles of both loci give identical phenotypes involving a build-up of undifferentiated cells at the shoot meristem in the L1 and L2, so that the PZ is shifted away from the apex. *clv1;clv3* double mutants exhibit the same phenotype as strong single mutants, further suggesting their roles in the same pathway. The *CVL1* gene has been isolated and found to encode a potential receptor kinase which might relay positional information to specific cells of the SAM (Clark *et al.*, 1997) *CLV1* is expressed in the L3 of a central region that extends beyond the CZ (Clark *et al.*, 1997). Therefore, it is possible that CLV1 may act to perceive positional information from the PZ to result in differentiation, or it may perceive a signal from the CZ, where it may act to restrict proliferation. It is possible that *CLV3* might encode the ligand for the product of *CLV1*.

Analysis of double mutants of a weak allele of *stm* (*stm-2*) and *clv1* and *clv3* showed that the products of both loci act competitively to regulate the same processes (Clark *et al.*, 1996). *CLV1* acting to repress proliferation at the SAM, while *STM* acts to promote meristem formation and maintenance. Neither requires the full activity of the other and mutations at both loci dominantly suppress each other's phenotype, suggesting that they act competitively on a common target.

The *NO APICAL MERISTEM* (*NAM*) gene of petunia is a member of a gene family encoding a novel class of proteins (Souer *et al.*, 1996). *nam* mutant embryos form cotyledons which are fused at their petioles, but no true leaves are produced upon germination. The seedling's growth is arrested and it eventually dies. Following germination, large, highly vacuolated cells were formed in the position where the SAM would normally have developed. Since *NAM* is expressed in a ring surrounding the embryonic SAM, it may act in a non-cell autonomous manner to allow meristem formation in the embryo. It is also possible that it prevents the proliferation of the surrounding tissue and in the absence of NAM, the development is disrupted by over proliferation of adjacent cells. *NAM* is expressed in the embryo at a later stage than *STM* in *Arabidopsis* embryos (Long *et al.*, 1996), suggesting that the SAM has been initiated and that *NAM* is necessary for further development.

Infrequently, "escape" shoots would develop in *nam* mutants. These shoots develop from cells which look like a canonical SAM but are surrounded by the same large cells. Some of these escape shoots develop into mature plants, while others only form a few leaves. In the latter case, the vascular supply of these shoots is not connected to that of the hypocotyl and root. It is probable that escape shoots result from a group of cells being able to overcome the repressive action of surrounding cells in *nam* mutants, to organise themselves into a SAM. This is probably not due to any residual *NAM* activity, because it is observed in plants carrying likely null *nam* alleles, but to the partial redundancy of *NAM* function.

The flowers formed by these escape shoots contained abnormal floral organs with an increased number of petals formed in whorl 2, often fused to stamens in whorl 3. Ectopic antheroid organs formed between whorls 3 and 4. Variable abnormalities were also seen in whorl 4 organs. As a result, these escape shoots were male and female sterile.

In floral meristems, expression of *NAM* also predicted the boundaries of whorls 2 and 3. Later, there was expression between the two carpels. *NAM* went on to be expressed along the placenta edge to mark the boundaries of ovule primordia. During ovule development, expression of *NAM* again occurred in rings marking the sites where integuments would form.

Therefore, in postembryonic development, *NAM* is thought to act to mark boundaries of primordia before they are visibly separate from the meristem. It seems that *NAM* expression is required to prevent these regions from becoming primordia too. Vincent *et al.*, (1995) showed that in *Antirrhinum*, there is no lineage restriction between floral organ whorls at early stages. Once restrictions have been put in place, organ identity genes are expressed. *NAM* may act to influence the orientation or rate of growth in these boundary regions, thus preventing cell lineages from contributing to more that one whorl. In *Antirrhinum*, *FIMBRIATA* (*FIM*) is also expressed in rings marking the boundaries of floral organs (Simon *et al.*, 1994), suggesting a role linking the expression of organ-identity genes to the restriction of cell lineage.

There is weak *NAM* expression in the stem and in young leaves, as well as expression in inflorescence meristems in rings marking the boundaries of bracts and floral meristems as they formed. However, there is no *nam* mutant phenotype in these sites, possibly due to the functional redundancy of *NAM*.

Organ Formation

Lateral organs such as bracts and petals, are thought of as being modified leaves, so that the leaf may represent a developmental pattern from which other organs have evolved.

Leaves are initiated by groups of cells within the flanks of the vegetative apical meristem. These cells are found in all three cell layers of the meristem. Leaf initiation involves changes in polarity and the rate of cell division and expansion within these initial cells. What distinguishes leaf fate from stem fate? The leaf grows in a novel axis, laterally away from the stem. The leaf is a determinate lateral organ unlike the stem which has a SAM at its apex so that it has the potential for indefinite growth. Leaves are usually dorsoventrally flattened, unlike the stem which is usually radially symmetrical. Specialised cell types differentiate in layers perpendicular to the dorsoventral axis. This flattening is usually seen as primordia emerge from the meristem in maize, while in *Antirrhinum* asymmetry can only be seen following emergence. Leaves also exhibit a proximodistal axis which becomes obvious as organ initial cells form a primordium which grows away from the meristem. Morphological differences are seen along this axis, for example, the proximal petiole is much narrower than the distal leaf lamina.

Differences along their proximodistal and dorsoventral axes are common to all lateral organs and are seen early in organ development. Perhaps the definition of these two axes in lateral organ initials coincides with their differentiation from the SAM.

To further understand the commitment of meristem cells to lateral organ fate, it was necessary to identify a gene which was expressed solely in organ initials cells. The only markers for lateral organ fate so far were (i) genes expressed in undetermined cells of the meristem which were downregulated in organ initial cells, like *STM*, and (ii) genes expressed in the border region between meristem cells and organ initial cells, like *NAM* (Clark *et* al., 1996; Long *et al.*, 1996; Souer *et al.*, 1996). It is shown here that the timing and pattern of *PHAN* mRNA expression coincides with the determination of lateral organ identity.

The leaf primordia of the monocot maize, are already dorsoventrally flattened at emergence and encircle the SAM, in contrast to *Antirrhinum* and other dicots where primordia are initially radially symmetrical (Steeves and Sussex, 1989). It has been shown that the down regulation of *KN1* expression in the SAM correlates with the initiation of a leaf primordium, while the related gene, *ROUGH SHEATH 1* (*RS1*), is expressed at the basal limit of the primordium (Jackson *et al.*, 1994), similarly to *STM and NAM* respectively, indicating that similar domains are specified within the SAM of maize, *Arabidopsis* and petunia . The maize leaf is composed of three domains. Proximally is the sheath, which acts to support the plant by wrapping round the culm, and distally the blade. At the boundary of blade and sheath is the ligular region which is composed of the two wedge shaped auricle which act as a hinge allowing the leaf to bend, and the ligule, which is a region of epidermal tissue that grows at right angle to the leaf, sealing the blade to the culm. The first feature of the leaf primordium is three regions of dorsal cell types which correspond to the three regions of the mature leaf (Sylvester *et al.*, 1990). Early in development, the blade sheath boundary, where the ligular region will form, takes up a relatively large area (Sylvester *et al.*, 1990). Taken together with the fact that no mutations remove the blade-sheath boundary, Freeling *et al.* (1992) argued that the establishment of this boundary had a fundamental role in maize leaf development. There are many mutations that exhibit a blade to sheath transformation which prompted Freeling (1992) to propose a maturation schedule hypothesis. This states that the domains of a wild type leaf pass through competency stages such that the final fate is determined by the stage at which the signal to differentiate is received. Differentiation occurs basipetally from tip to base and also laterally from the midrib to margin. So the midrib will enter the schedule first and will be competent to form blade, before proximal and marginal cells. Another way of viewing it, is to think of the definition of spatial domains rather than maturity. Thus, mutations which delay competence to respond or disrupt signalling will result in blade-sheath or central-marginal transformations.

The growth of the ligule in a novel axis at the boundary of sheath-blade domains, is analogous to the proliferation of the lamina at right angles to the dorsal and ventral boundaries in dicot leaves (Hudson and Waites 1998). Two genes, *liguleless1* (*lg1*) and *liguleless2* (*lg2*) are involved in the formation of the ligular region. *lg1* mutants have no auricles and no ligules on the first 10 leaves, after which ligule like organs are seen (Becraft *et al.*, 1990; Sylvester *et al.*, 1990). *liguleless2* mutants have no ligule or auricle on the first two leaves after which there was a gradual restoration of ligule and auricle, till they strongly resemble wild type (Harper and Freeling, 1996). Thus, recessive mutations in both these unlinked genes result in the loss of ligule and auricle. The presence of rudimentary ligules in the older leaves of both mutants suggests that there may be an 'adult ligule' program which does not require either gene, or it may reflect the redundancy of the expression patterns of both genes in older leaves (Harper and Freeling, 1996). Mosaic analysis has shown that *lg1* function is cell autonomous (Becraft *et* al., 1990) This was shown by the simultaneous somatic loss of a cell autonomous marker and a dominant gene of interest. If the domain of loss of marker coincides with the domain of the gene of interest's mutant phenotype, then it is acting cell autonomously, while if the two domains do not coincide, it can be said to be acting non-cell autonomously. Using mosaic analysis, the *lg2* function was shown to be non-cell autonomous, since *lg2-R/deletion* sectors did not show a loss of ligule or auricle (Harper and Freeling, 1996). The double mutant phenotype suggests that *LG1* and *LG2* interact in the same pathway since the *lg1-R* and *lg2-R* (genetic null alleles) functions were dosage dependant upon each other (Harper and Freeling, 1996). The dosage sensitivity suggests that LG1 and LG2 may act near to each other, temporally, in the ligule/auricle developmental pathway. Both *lg1-R* and *lg2-R* phenotypes show a disturbed blade-sheath boundary (Harper and Freeling, 1996; Becraft *et al.*, 1990), suggesting that both LG1 and LG2 functions interact to form a normal blade-sheath boundary. This boundary is set up at a very early stage in plastochron two or three of the development of the leaf primordium (Sylvester *et al.*, 1990; Freeling 1992).

Therefore, it seems that in both monocot and dicot systems, there is proliferation in a novel axis at the boundaries of different cell types. In support of this view, patches of ectopic *kn1*-like gene expression result in the formation of sheath tissue on the blade,

with ligule and auricle tissue at the boundaries (Sinha and Hake, 1994). It must be noted that whilst there are similarities in leaf development between dicots and monocots, the two developmental programs may have developed independently of each other. Zimmermann's telome theory (1965) suggests that leaves evolved from dichotomous branched systems. The most primitive angiosperm probably had lobed leaves as suggested by the presence of three primary leaf traces in the primitive members of the class (Sinnott, 1914). Anatomical and developmental evidence indicates that the original monocots had leaves which were relatively small, simple structures due to a reduction of the ancestral type (Arber, 1925). This reduction may have involved the loss of the lamina leaving a radial petiole, not unlike modern rushes and onions. A phyllode is a flattened petiole and it has been suggested that the maize leaf blade is a phyllode, being petiole derived (Arber, 1925). This would account for the parallel venation found in the monocots which is what would be expected if a radial petiole was flattened. The dicot plantain leaf, which resembles that of monocots in having parallel venation, is probably derived independently from the petiole (Q. Cronk, pers. comm.). Thus, it has been hypothesised that in response to a change to a more aquatic, marsh environment, the monocot leaves became unifacial through the loss of lamina, i.e. produced radially symmetrical leaves resembling those of modern rushes. The presence of lamina in monocots today can be accounted for by another change in environment to a drier one, such that the gain of a dorsoventrally flattened lamina was advantageous. To do this, monocots formed a 'pseudo lamina' through the proliferation of the petiole. Some monocot leaves have a unifacial rudimentary tip at the distal apex of the blade, which is equivalent to the dicot lamina. Therefore, in maize, *PHAN* homologues may result in the lateral proliferation of the petiole as opposed to the lateral proliferation of the petiole and lamina by *PHAN* in *Antirrhinum*. However, Stebbins (1974) rejects this theory and favours the hypothesis that both are derived from common ancestor, which had a flattened lamina with netted venation and a less distinct petiole, through modifications, not involving the loss of true lamina, taking place differentially within the monocots and dicots. Therefore, Stebbins argues that the dorsal lamina surfaces of both monocots and dicots resulted from the same ancestral type.

Thus, if the phyllode theory is correct, the Maize leaf can be thought of as being analogous to the leaves of *Antirrhinum* or *Arabidopsis* although the two leaf structures probably resulted from convergent evolution. Since there are genes involved in leaf development that are homologous between *Antirrhinum*, *Arabidopsis* and Maize, it is likely that these genes were present in the ancestral form and now regulate the different leaf developmental programs.

This infers that the regulatory networks governing the initiation of lateral organs occurred early in plant evolution and has been conserved between these distantly related species.

PHAN was shown to encode a transcription factor of the MYB family. Therefore the structure and function of MYBs is reviewed here.

c-MYB

Introduction

c-MYB, a nuclear proto-oncogene, encodes a transcription factor that is essential for the proliferation of haematopoietic precursor cells (reviewed in Shen-Ong, 1990; Thompson and Ramsay, 1995). Proto-oncogenes were first identified as the cellular cognates of retroviral growth-promoting transforming genes. Oncogene-encoded proteins disrupt cellular metabolism in a number of ways, e.g.. acting as growth factors, intracellular components of signal transduction pathways, and by controlling gene expression so the expression of oncogenes can lead to the deregulation of cell proliferation. Cellular homologues, which have been identified for many oncogenes, play a role in cell growth and differentiation. Involvement in such basic functions is reflected by the high conservation of these genes between vertebrates.

c-MYB encodes a protein that is approximately 75 kDa in chicken and mouse, and approximately 80 kDa in humans. It consists of three functional units, the DNA binding domain, the transactivation domain and negative regulatory elements (Figure 7). All three domains are vital for the correct functioning of MYB.

The two avian retroviral forms of v-MYB are encoded by the avian myeloblastosis virus (AMV) and E26 (reviewed by Moscovici *et al.*, 1985), (Figure 7). The *v-MYB* oncogene causes monocytic leukemia in chickens by transforming myeloid cells. It appears to have resulted from retroviral insertional mutagenesis, recombination with *c-MYB* and then rescue by a replication-competent helper virus (Baluda and Reddy, 1994). The resultant v-MYB protein is a doubly truncated, de-repressed form of c-MYB.

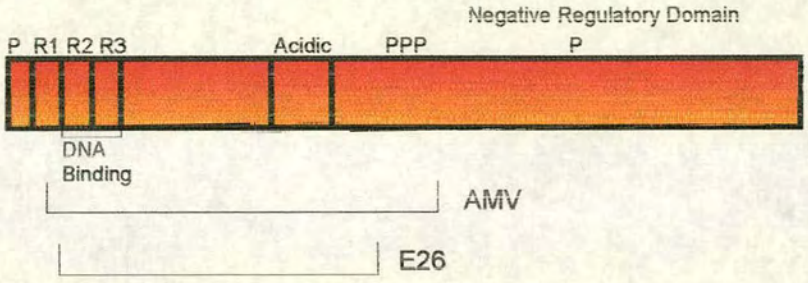


Figure 7. Schematic diagram showing the functional domains of c-MYB (Modified from Lipsick, 1996).

P = phosphorylation sites; AMV = extent of c-MYB in v-MYB of avian myeloblastosis virus; E26 = extent of c-MYB in Gag-Myb-Ets of E26 leukemia virus.

The MYB Domain

The DNA binding domain is at the amino terminal end and consists of three repeat sequences, termed, R1, R2 and R3 (Figure 7), which have been highly conserved. Each repeat has three conserved tryptophans at 18-19 amino acid intervals (Anton and Frampton, 1988). NMR analysis and molecular modelling predict that each repeat is made up of three helical bundles.

The minimal specific DNA binding region is confined to the homologous R2 and R3 repeats (Luscher and Eisenman, 1990). The consensus c-MYB DNA recognition sequence is PyAACG/T, where R3 recognises the AAC sequence while R2 recognises the downstream flanking sequence less stringently (Tanikawa *et al.*, 1993). The NMR derived solution structure of R3 was found to have three α-helices with two turns (Ogata *et al.*, 1992). The conserved tryptophans form a hydrophobic core and play an important role in sequence specific DNA binding (Kanei-Ishii *et al.*, 1990; Saikumar *et al.*, 1990). Helices 2 and 3 form helix turn helix (HTH) structures which differ from the conventional HTH motif. The differences lie in the turn which is a residue longer and contains a proline residue, not found in a conventional turn. The relative orientations of helices 2 and 3 are also distinct from canonical HTH structure. The helices 1 and 2 of R3 are aligned obliquely with a short turn, and helix 3 lies nearly perpendicular to helix 1.

Tanikawa *et al*, (1993) have shown that the first repeat promotes stability of the DNA-MYB complex, while the N-terminal acidic region acts to reduce this stability by blocking binding (Dini and Lipsick, 1993). This region contains two serine residues which, when phosphorylated by casein kinase II, can further inhibit the binding of MYB to DNA (Lusher *et al.*, 1990) by locally increasing the negative charge. Deletion of the N-terminal region activates the *c-MYB* proto-oncogene, consistent with its role in negative regulation. R1 and the N-terminal region may also play a role in differential binding at particular binding sites. The sequence specificity of c-MYB is quite low so that it binds to multiple sequences. However,

phosphorylation of the N-terminal region inhibits binding only at low affinity binding sites, leaving binding at high affinity sites unaffected (Lusher *et al.*, 1990). Thus, it seems that the N-terminal region may only block binding at specific sites, to affect non-specific binding. Interestingly, plant MYBs lack this motif, suggesting that they do not share this mechanism of regulation. However, plant MYBs MYB305 and MYB340 can still be regulated by phosphorylation (Moyano *et al.*, 1996).

A single cysteine (Cys43) is conserved in a region in R2 predicted to form the DNA recognition helix (Frampton *et al.*, 1991). However, Jamin *et al.*, (1993) found that this region adopted a disordered flexible structure in solution. Myrset *et al.*, (1993) hypothesised that upon binding to DNA, this disordered structure forms the recognition helix which generates the full HTH motif. They found that Cys43 was accessible to alkylation when in the free protein, but not when in the protein-DNA complex. Two explanations for this are that (i) the DNA protects the Cys43 which is in the DNA-binding site, or (ii) there is a conformational change upon binding to DNA which results in the Cys being protected from alkylation in the interior of the protein. When the NMR-derived structure of R3 (Ogata *et al.*, 1992) is used to model the homologous R2 repeat, Cys43 is placed in the hydrophobic core of R2, which is consistent with the second explanation. In addition, Myrset *et al* found from mutational analysis of Cys43, that the critical feature of this residue was the presence of a hydrophobic side chain which would allow it to move to the R2R3 interior upon binding to DNA. Spectroscopic studies indicated that there was tighter folding of R2 upon binding to DNA. The differences between the spectra of Cys43 mutants in the native and DNA-bound form decreased, as the hydrophobicity of the side chains increased. This indicates that high hydrophobicity at this position induces a conformational change nearer to the bound state of the protein. In addition, their results confirmed that the highly oxidizable Cys43 might function as a molecular sensor for a redox regulatory system, which would turn specific DNA binding on or off via a DNA induced conformational change in R2. When the Cys43 was oxidised, the fluorescence spectrum was similar to that seen for mutant proteins with hydrophilic amino acid substitutions. Therefore the protein structure was proposed to have opened up due to oxidation and the proper conformational changes that occur

upon binding to DNA, were not able to take place. Since oxidation is reversible, the Cys43 could act to regulate specific DNA-binding depending on its redox state. It has not been shown if such a post-translational regulatory system is important for the biological function of MYB.

Transactivation Domain

The transactivation domain (Figure 7) is a region of acidic residues and is necessary to activate reporter gene expression (Ibanez and Lipsick, 1990). The activity of this region is inhibited by the carboxy terminus of MYB. Without this C-terminal sequence, MYB displays improved DNA binding affinity (Ramsay *et al.*, 1992) and increased the rate of primary cell proliferation and transformation of haemopoietic cells *in vitro*, when compared to full-length MYB (Grasser *et al.*, 1991). The two non-overlapping sub-domains that make up this negative regulatory domain can independently inhibit transcriptional activation, acting separately or interacting with each other (Dubendorff *et al.*, 1993). A leucine zipper structure is present within this negative regulatory domain (Biedenkapp et al., 1988), and Kanei Ishii *et al.* (1992) found that point mutations in this motif promoted transactivation and transformation. Leucine zippers usually mediate protein-protein interactions, which suggests that the domain might act in one of two ways. Firstly, c-MYB might homodimerise at the leucine zipper structure so blocking DNA binding or transactivation, or secondly, other cellular molecules may bind to the zipper to inhibit MYB function. Proteins that bind to the leucine zipper have been identified (Favier and Gonda, 1994), but it is unclear whether they form transcription complexes with MYB or if they bind to inhibit MYB function.

There are several phosphorylation sites near to the negative regulatory region. Studies *in vitro* suggest that the activity of c-MYB may be regulated by phosphorylation in this region. Aziz *et al.* (1993) demonstrated that c-MYB is phosphorylated in cell lines at sites which are targets for $p42^{MAPK}$ *in vitro*. They found that serine528 is phosphorylated *in vivo*. The substitution of alanine for Ser528 resulted in an increase in transactivation of reporter construct in cell culture. This

indicated that phosphorylation of Ser528 negatively regulates c-MYB activity. Dubendorff *et al.* (1993) showed that in chicken c-MYB, the carboxy-terminus could suppress transactivation by MYB in *trans*. The suppression could result from direct interaction by the carboxy-terminal containing the Ser528 and the transactivation domain, or by another protein which interacts with the transactivation domain, but is stabilised by the phosphorylated Ser528 domain. The carboxy-terminus has consensus sequences for a number of kinases, so that it may act to integrate many signal transduction pathways through different phosphorylation/dephosphorylation events.

Maize MYB-and MYC-Related Transcription Factors

The first transcription factor with a known function to be isolated in plants was the maize *C1* gene. Its product showed homology to the DNA-binding domain of the vertebrate c-MYB. C1 protein plays a role in controlling phenylpropanoid metabolism. More specifically, it activates the transcription of genes that encode enzymes involved in the biosynthesis of anthocyanin in the outer layer of maize seed endosperm, the aleurone (Paz-Arez *et al.*, 1987). The *Pl* gene (which is homologous to *C1*) controls anthocyanin production in the remaining parts of the maize plant. The maize genes *R* and *B* are MYC-related factors also involved in anthocyanin production. The characteristic feature of MYC-related transcription factors is the basic helix loop helix (bHLH) domain, which functions in protein-protein interactions through the formation of homo- or heterodimers at the HLH domain (Lassar *et al.*, 1989). This brings together two basic regions to form a functional DNA-binding domain which allows efficient target DNA recognition by subunit dimerization (Voronova and Baltimore, 1990). Both C1 and Pl act together with R and B respectively in an obligate fashion. For example, C1 only increases anthocyanin production in transgenic *Arabidopsis* when co-expressed with R. C1 also interacts with B in yeast, showing that the regulation of anthocyanin pigmentation in maize may involve the direct interaction between two different classes of transcription factors. The C-terminal transactivation region of C1 can be fused to the DNA binding domain of the yeast transcriptional activator GAL4 and still function as a transactivation

domain in yeast (Goff *et al.*, 1991). If the transcriptional activation domain of GAL4 is substituted for the C-terminal region of C1, the resulting fusion protein is still capable of transcriptional activation in transformed yeast, but only in the presence of B (Goff *et al.*, 1991) suggesting that B and C1 interact directly to regulate their target promoters. Deletion studies have shown that the bHLH domain of B and most of the C-terminal region are not required for transactivation in transformed maize cells. What is important is the N-terminal region which binds to the MYB domain of C1 to form a transactivation complex containing both the bHLH DNA-binding and subunit dimerization domain of B as well as the MYB DNA-binding domain of C1 (Goff *et al*, 1992). The formation of a MYB-MYC complex is thought to increase the specificity and/or affinity of the binding domains supplied by each protein. Goff *et al*, favour the idea that the bHLH deletion derivatives of B are partially active due to N-terminal interaction with C1 which anchors the complex to the target promoter via the MYB DNA binding domain. There is no block conservation of bHLH and MYB consensus DNA binding sites in promoters of structural genes of the anthocyanin biosynthetic pathway. So, the regulation of different structural genes may be through differential dependence on these two classes of transcription factors, since there is no evidence for these structural genes being regulated as a block.

Other Plant MYB Transcription Factors

There are three cellular members of the vertebrate MYB family, all playing roles in cellular proliferation in actively dividing cells, prior to division. They also recognise similar target motifs. Following the isolation of *C1* from maize, over a hundred other *MYB* genes have been identified in higher plants (reviewed by Martin and Paz-Ares, 1997). They have been assigned functions in secondary metabolism, cellular morphogenesis, signal transduction and plant growth regulation (Figure 8). Within these groups, there are sub-families with overlapping function, as is the case with vertebrate MYBs.

MYB Protein	Species	Function or implied function
C1	*Zea mays*	Phenylpropanoid metabolism
Pl	*Zea mays*	Phenylpropanoid metabolism
P	*Zea mays*	Phenylpropanoid metabolism
ZmMYB1	*Zea mays*	Phenylpropanoid metabolism
ZmMYB38	*Zea mays*	Phenylpropanoid metabolism
AmMYB305	*Antirrhinum majus*	Phenylpropanoid metabolism
AmMYB340	*Antirrhinum majus*	Phenylpropanoid metabolism
MIXTA	*Antirrhinum majus*	Cell shape
GL1	*Arabidopsis thaliana*	Cell shape
AtMYB1	*Arabidopsis thaliana*	Hormone response
AtMYB2	*Arabidopsis thaliana*	Hormone response
PhMYB1	*Petunia hybrida*	Cell shape
PhMYB3	*Petunia hybrida*	Phenylpropanoid metabolism
PhMYBAn2	*Petunia hybrida*	Phenylpropanoid metabolism
GAMYB	*Hordum vulgare*	Hormone response

Figure 8. Table showing examples of plant MYBs and their functions. (Modified from Martin and Paz-Arez, 1997)

The structural characteristics of vertebrate MYBs are conserved in plants, except for the absence of R1, leaving two imperfect repeats, R2 and R3 of about 50 residues each. There are exceptions which contain only one of these repeats (Baranowskij *et al.*, 1994). Some fungal MYBs also have just two repeats (Ohi, *et al.*, 1994, Wieser and Adams, 1995) suggesting that R2/R3 may be the ancient DNA binding domain structure, with animals having gained an extra repeat. Therefore, information gained from the study of vertebrate MYBs may be directly applicable to plant MYBs.

MYBs can bind common target motifs. An example in plants is provided by AmMYB305 and AmMYB340 which are two flower specific proteins from *Antirrhinum majus*. They differ only in 4 amino acids of the deduced sequence of their two DNA binding domains (Jackson *et al.*, 1991), suggesting that they may

recognise a common target motif. DNase I protection and competitive DNA binding studies confirmed that both proteins compete for the same promoter sequence of the genes encoding phenylalanine ammonium lyase (PAL), the first enzyme of phenylpropanoid metabolism, and two other enzymes (CH1 and F3H) involved in flavonoid metabolism. However more of the MYB305 protein is able to bind to the target promoter *in vivo* due to differential phosphorylation of the two proteins. Conversely, transforming tobacco leaf protoplasts with genes encoding each protein shows that MYB340 activates target promoters more strongly than does MYB305. When co-transformed, the activation of target promoters by MYB340 is inhibited by MYB305, suggesting a competitive interaction between the two proteins. The expression patterns of each of these genes overlap, but with MYB340 being expressed slightly earlier in young buds (Jackson *et al.*, 1991) It is therefore possible that MYB340 is expressed early to provide a rapid activation of target gene expression, after which MYB305 is expressed to bring the level of gene expression down to a lower, maintenance level. So, the relative amounts of each protein and their different activation abilities determine the activation of their targets (Moyano *et al.*, 1996). A further level of control may be imposed by interaction with other proteins, as shown by the relationships between C1 and B in maize.

The binding specificities of plant MYBs vary greatly between each other and from vertebrate proteins (e.g. Solano *et al.*, 1994). While plant MYBs have a homologous DNA binding domain in common, differences in base-contacting residues and in the overall context of their MYB domains, produce different DNA binding specificities (Solano *et al.*,1995a and Solano *et al.*, 1995b).

How is MYB activity regulated in plants? There is considerable evidence from the differential accumulation of mRNA in an organ specific and temporal manner, for transcriptional control. An example is the *MIXTA* gene from *Antirrhinum* which is involved in the control of cell shape (Noda *et al.*, 1994). It is required for the development of the conical shaped cells of the petal epidermis and its expression is restricted to the petal lobes of older flowers.

Postranslational regulation is implicated through cellular redox potentials (Myrset *et al.*, 1993), phosphorylation, and protein-protein interactions. Because of the structural conservation between vertebrates and plants, similar forms of regulation may be expected in both systems. Cys43, proposed to function in a redox regulatory system of *c*-MYB (Myrset *et al.*, 1993) is conserved in plant MYBs, however redox sensitive regulation has yet to be demonstrated in plants, *in vivo*.

Many serine and threonine residues at the C-termini of plant MYBs are possible substrates for kinases and phosphatases, suggesting potential regulation of DNA binding or transactivation through phosphorylation, similarly to c-MYB (Luscher and Eiseman, 1990). The only evidence to support this is that AmMYB340 synthesised in yeast and *E. coli*, requires the addition of alkaline phosphatase to recover the high DNA binding affinity of the protein synthesised *in vitro* (Moyano *et al.*, 1996) which suggests that phosphorylation plays a role in inhibiting the activation ability of AmMYB340.

Evolution of MYBs

MYB proteins have been identified in all eukaryotic groups, implying that MYB-like DNA binding domains developed to regulate gene expression early in evolution. Sequence comparisons between different MYB proteins shows that there is greater conservation between the equivalent repeats of different proteins than between the repeats in the same protein. This indicates that the duplication events that generated these tandem repeats occurred prior to the eukaryotic radiation which gave rise to animals, major land plants and fungi. Nearly all plant MYBs contain R2 and R3, many having a conserved proline in their R2. Sequence comparisons also show that multiple *MYB* genes in plants seem to have arisen from gene duplication, prior to the divergence of monocots and dicots. However, the Myb repeats of ATMYB1 from *Arabidopsis* resemble those of animals more than those of other plant genes and lack the R2 proline unique to plants. This suggests a model where the MYB-related genes of flowering plants came about by duplication of an ATMYB1-like progenitor, acquisition of a proline substitution in one of these genes, and then rounds of

duplication of both sets of genes (Lipsick, 1996). Slime moulds are thought to have diverged before the major eukaryotic radiation (Knoll, 1992). Surprisingly, the slime mould, *Dictyostelium discoideum* contains a MYB with three repeats (Stober-Grasser *et al.*, 1992), which suggests that the three repeat MYB was present in the common ancestor of slime moulds and animals and therefore also in ancestral plants and fungi. This three repeat MYB has presumably been lost from plant and fungal lineages.

The wide deployment of MYBs in plants suggests that as the eukaryotes diverged, plants developed novel functions and used MYBs extensively to control the expression of these novel structural genes (Martin and Paz-Ares, 1997). So, following the divergence of plants from other eukaryotes, there was a loss the R1, then genome duplication to increase the number of MYBs and subsequent divergence of *MYB* genes.

Materials and Methods

Plant Material

DNA Manipulation

in situ Hybridisation

Plant Material

Antirrhinum majus stock lines

JI. 75

The wild type line, JI. 75 was produced at the John Innes Institute, Norwich, UK from a *palida* revertant plant (Pal^{+} / *pal*^{recurrens}) which showed occasional pale sites on a red petals. This was proposed to be due to the large number of transposons that are active in this genetic background (Harrison and Carpenter, 1979). Consequently, it has been used in large scale mutagenesis experiments (Carpenter and Coen, 1990)

phan^{ambigua}-250G and *phan*^{antiqua}-249G

Both mutants *phan*-250G and *phan*-249G were originally isolated by Baur (1926) and were obtained from the Zentralinstitut fur Genetik und Kulturflanzenforschung, Garteslaben, Germany, and inbred as homozygotes for at least a further three generations. Both lines are homozygous for the semi-dominant *Eluta* mutant allele and for the *delila* allele which limit anthocyanin pigmentation in the flower.

phan-607 and *phan*-709

The *phan*-607 and phan-709 mutants were found in large scale mutagenesis experiments using JI. 75 (Carpenter and Coen, 1990) and were provided by the John Innes institute, Norwich, UK. Both were shown to carry single recessive *phan* mutations.

phan-552

This mutant was identified in a targeted mutagenesis programme (Waites and Hudson, 1995) and was produced by crossing JI. 75 with *phan*-249G. The new *phan*-552 mutant was heterozygous, *phan*-552 / *phan*-249G. One mutant allele had been inherited from the mutant parent, *phan*-249G, and the other from a new Tam 4

insertion into a gamete from the wild-type parent, line JI. 75. The *phan*-552 mutation was germinally unstable (no evidence of somatic instability) and thus allowed the cloning of the *PHAN* gene by transposon tagging.

Plant growth conditions

Seeds were grown in Levington M3 potting compost. Seedlings were repotted twice before they reached maturity.

Vegetative cuttings were taken from mature plants by removing healthy shoot tips and dipping the cut ends in hormone rooting powder (PBI Roota). The cuttings were allowed to root under a water misting unit in the greenhouse. Typically three or four weeks were sufficient for roots to become established after which the cuttings were transplanted into M3 compost.

Pollination was carried out by transferring pollen (using toothpicks) from stamens to the stigmas of open flowers when self pollinating, and on to stigmas of emasculated flowers, when cross pollinating.

Plants were grown in greenhouses at an average temperature of 20°C. In winter, daylight was supplemented with light from metal halide lamps. Plants were also grown in growth cabinets at 15°C, 20°C and 25°C in 16 hr light - 8 hr dark cycles, with light from metal halide lamps.

DNA Manipulation

***Escherichia coli* strains and plasmid vectors**

JM101 (*sup*E, thi, Δ(*lac-pro*AB)rpsL, [F', *tra*D36, *pro*AB, *lac*Iq, *lac*ZΔM15])

DH5α (*sup*E44, Δ*lac*U169 (f80*lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gry*A96, *thi*-1, *rel*A1.

Both strains were used as plasmid hosts allowing recombinant plasmids derived from pBluescript (Stratagene) to be identified using a process known as *alpha*-complementaion.

DNA inserts were subcloned into pBluescript plasmid vectors. These plasmids contain a selectable marker (ampicillin resistance) and a polylinker inserted into the coding region of a *LacZ* gene fragment which encodes the amino-terminal portion of the β-galactosidase enzyme. The plasmid gene product alone is inactive but gives enzyme activity when combined with the product of the *LacZ* DM15 allele, which encodes an amino-terminal defective form of β-galactosidase. Insertion of DNA into the plasmid polylinker disrupts *LacZ* expression making recombinant plasmids unable to perform *alpha*-complementation with the appropriate bacterial host. The active β-galactosidase can catalyze the removal of a galactose residue from the colourless X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside), converting it to a blue derivative. This makes host bacteria containing recombinant plasmid (white colour) readily distinguishable from those containing non-recombinant plasmid (blue colour) when plated on X-Gal plates. Other properties that made the pBluescript plasmids useful were: an origin of replication derived from bacteriophage M13 which allows the isolation of single stranded DNA; binding sites for the M13 universal primers, conveniently placed for sequencing and PCR of DNA inserts; and bacteriophage T7 and T3 RNA polymerases adjacent to the polylinker, facilitating the synthesis of RNA from the DNA inserts.

Mini Gel Electrophoresis

To visualise small quantities of DNA, small miniscus gels were poured on thin glass plates measuring 15 cm x 6 cm. 25 ml of 0.7% agarose (w/v) in 0.5 x TBE with 10μg/ml of ethidium bromide was used. Prior to loading, 0.1 volumes of gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) Ficoll type 400) was added to the sample of DNA. A potential gradient of 10 V/cm was used to size fractionate the DNA for approximately 30 minutes, after which the DNA was visualised on a UV transilluminator.

Agarose gel electrophoresis

To separate larger quantities of DNA, 600 ml of 0.7% agarose (w/v) in TBE was used in a gel former. As for mini gels, gel loading buffer was added to the DNA solution before loading and electrophoresed at a potential gradient of 3 V/cm overnight. The gels were then stained in ethidium bromide and the DNA could then be visualised using a UV transilluminator.

***Antirrhinum* Genomic DNA Extraction**

2-4 g of young leaf material was collected in foil packets and frozen in liquid nitrogen. A chilled coffee grinder was used to grind the frozen material to a fine powder. The resulting pale green powder was mixed with 20 ml of DNA extraction buffer (3 x SSC , 0.1 M EDTA, 0.1 M sodiumdiethyldithioncarbonate) in a 50 ml Falcon tube. 5 ml of 10 % (w/v) SDS was added and the tube shaken vigorously to mix the contents. 20 ml of chloroform were then added and the mixture was again shaken vigorously. The mixture was centrifuged in a Mistral centrifuge at 3600 rpm for 5 minutes. The aqueous layer was transferred to a fresh tube and a half volume of phenol and chloroform (1 : 1) was added, mixed thoroughly and centrifuged as before. The aqueous layer was removed to a fresh tube and extracted with chloroform. The aqueous layer was again removed to a fresh tube and an equal volume of ethanol was added. This solution was then centrifuged for 10 minutes to pellet nucleic acids. The ethanol was discarded and the pellet was dissolved in 5 ml of TE with 5µl of 10 mg/ml RNaseA and incubated for 10 minutes at 37°C. Following the removal of RNA, 0.7 ml of 5 M NaCl and 5.7 ml of CTAB solution (2 % (w/v) hexadecyltrimethylammonium bromide, 50 mM Tris pH8.0, 10 mM EDTA) were added to precipitate the DNA. The DNA was pelleted by centrifugation. The pellet was washed in 70 % ethanol (v/v) / 0.5 M NaCl for at least an hour at room temperature or overnight at 4°C. The DNA was again pelleted and allowed to air dry. The pellet was then dissolved in 150 - 500 µl of TE depending on size.

Small scale preparation of plasmid DNA from bacterial culture by alkali lysis

1.5 -3 ml of overnight bacterial culture was centrifuged at 1300 rpm in a benchtop mini centrifuge for 30 seconds. The supernatant was discarded and the pellet of

bacterial cells was resuspended in 100 µl lysis buffer (50 mM glucose, 25 mM Tris pH8.0, 10 mM EDTA) so that no bacterial clumps remained. 200 µl of freshly made 0.2 M NaOH, 1% (w/v) SDS was added. 100µl of an ice cold solution of 3 M Potassium acetate and 2 M acetic acid was added and then incubated on ice for 5 minutes. The resultant white precipitate was pelleted by centrifuging for 3 minutes. The supernatant was removed to a fresh tube, extracted with phenol/chloroform and the nucleic acids were precipitated with ethanol. The pellet was washed in 70 % (v/v) ethanol, air dried and dissolved in 20 µl of TE plus 5 µl of 10 mg/ml RNaseA.

Measuring DNA concentration

The DNA was diluted 1 : 100 in a total volume of 1ml and the absorbance measured in a UV spectrophotometer at 260nm. The absorption spectrum of DNA is such that the absorption at 280 nm should be approximately half that at 260 nm. Protein impurities raise the absorption as a whole so the difference between absorbance at 260 nm and 280 nm is doubled to provide a better estimation of DNA concentration in the following calculation.

DNA concentration (mg/ml)= $2(A_{260} - A_{280})/20 \times 1000/10$

Restriction endonuclease digestion of DNA

5 µg of genomic DNA was digested in a total volume of 25 µl along with 2.5 ml of 10 x manufacturer's enzyme buffer, 1.5 µl restriction enzyme (15-18 units) and 1.25 µl of 1 µM spermidine chloride. Digestion proceeded at 37°C for about 2 hours.

For plasmid DNA digestion, the spermidine was omitted and the reaction carried out in a volume of 50 µl. Digestion was usually completed in 1 hour at 37°C.

Isolation of DNA fragments from agarose gels

Following size fractionation on an agarose gel, the required fragment was visualised on a UV transilluminator and cut out of the gel using a scalpel. The fragment was placed on a bed of glass beads within a 0.75 ml Eppendorf tube which had a hole in its base. This was placed inside a larger 1.5 ml Eppendorf tube and the two tubes

centrifuged at 13000 rpm in an MSE microfuge for 2 minutes untill the liquid had been removed from the gel slice. The liquid collected in the base of the larger tube. The DNA was then purified from the eluate using a GeneClean II kit (Bio 101.Inc) as per the manufacturer's instructions.

Ligations

Ligation into plasmid vectors was made using a three-fold molar ratio of insert to vector in a small volume (typically 5 ml) with 1x manufacturer's ligation buffer and 0.5U/ml of T4 DNA ligase (NBL). The reaction was allowed to proceed at 15°C overnight.

Bacterial Transformation

1 ml of a fresh overnight culture of DH5α or JM101 was used to innoculate 100 ml of L broth. The culture was grown till the absorbance at 550 nm was 0.45. The culture was chilled on ice for 15 minutes and centrifuged in 50 ml Falcon tubes at 4°C and 6500 *g* for 10 minutes. The pellet was resuspended in 4 ml of ice cold TFB1 (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% [v/v] glycerol, pH 5.8 using acetic acid). This suspension was centrifuged as before and resuspended in 4 ml ice cold TFB2 (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% [v/v] glycerol, pH 6.5 using KOH). The cells were aliquotted on ice and frozen in liquid nitrogen. The competent cells were stored at -70°C.

Each 100 ml aliquot of cells was thawed on ice for 10-15 minutes and then incubated with plasmid DNA on ice for a further 20 minutes. The cells were then heat shocked at 42°C for 90 seconds, briefly chilled on ice and allowed to recover in 0.5 ml of LB (Luria-Bertani medium, (1% Bacto-tryptone, 0.5% Bacto-yeast extract. 1% NaCl, pH 7.5 with NaOH)) at 37°C for 1 hour. The cells were then spread on LB plates (LB containing 1.5% Bacto agar) with appropriate antibiotic selection and grown overnight at 37°C.

Precipitation of DNA with ethanol

0.1 volume of 3 M Sodium acetate was added to the DNA solution and then 2.5 volumes of ethanol. The DNA was incubated at -20°C for 30 minutes and pelleted by centrifuging for 10 minutes. The DNA pellet was washed with 70% (v/v) ethanol and air dried. The DNA was dissolved in an appropriate volume of TE.

Phenol/Chloroform extraction

Phenol was first saturated with 10 mM Tris pH 8.0, mixed with an equal volume of chloroform and centrifuged to separate organic (phenol/chloroform) and aqueous layers. To extract proteins from DNA solutions, an equal volume of phenol/chloroform was added to the DNA solution. This is vortexed to mix and then centrifuged to give a upper aqueous layer containing DNA, which was removed to a fresh tube.

Polymerase chain reaction (PCR)

A Rapidcycler™ (Idaho Technology) was used to perform rapid temperature cycling based on heat transfer by hot air to samples contained in thin walled glass capillary tubes. The reactions contained 250-500μg/ml BSA to prevent surface denaturation of the enzyme. This was otherwise a problem due to the high surface area to volume ratio of the tubes which provided many sites for enzyme inactivation. Approximately 50 ng of template genomic DNA or 100 fg of plasmid DNA was used with buffer (50 mM Tris pH 8.3, 0.25 mg/ml crystalline BSA, 3 mM $MgCl_2$, 0.5% Ficoll 400 and 1mM tartrazine), 200 μM of each dNTP (deoxy nucleoside 5' triphosphate), 0.2 μM each of both primers and 0.2 U of *Taq* DNA polymerase (Bio Polythermase) in a total volume of 10 μl. Typically the reaction was predenatured at 94°C for 2 minutes, followed by 35 cycles of three temperatures (94°C to denature DNA, 45°C to anneal primers and 72°C to extend [extension time = 1 sec per 50 bp for products < 500 bp, or 1 sec per 25 bp for products > 2 kb]) and a final extension at 72°C for 2 minutes. The annealing temperature varied according to the melting temperatures of the primers. Products were then visualised with ethidium bromide on an agarose gel or purified on PCR purification columns (Qiagen).

Southern Analysis

Southern blotting

Following gel electrophoresis, the gel was stained with ethidium bromide (10 mg/ml) for 20 minutes and photographed on a UV transilluminator. It was then gently agitated in 0.25 M HCl for 10 minutes to allow the acid to depurinate DNA causing it to become nicked. This allowed larger fragments of DNA to be blotted efficiently. The gel was then gently agitated in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 20 minutes and then in neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.2) for 20 minutes. The DNA was transferred from the gel onto Hybond-N nylon filter (0.45 μm, Amersham) using 20 x SSC (1M NaCl, 100 mM Na citrate) as the transfer medium in an assembly similar to that described by Maniatis *et al* (1982). Following Southern transfer, the filter was washed briefly in 2X SSC and allowed to air dry before the DNA was crosslinked to the filter using 0.4 J cm^{-2} of UV light from a transilluminator.

Oligolabelling of DNA

25 - 50 ng of DNA in 15.5 μl of water was boiled for 10 minutes so that it became single stranded. It was then cooled by placing briefly on ice. 6 μl of oligolabelling buffer (250 mM Tris-HCl, pH8.0, 25 mM $MgCl_2$, 5 mM ß-mercaptoethanol, 2 mM each of dATP, dGTP, dTTP, 1 M HEPES, pH 6.0, 1 mg/ml random hexamers), 1 μl of bovine serum albumin (10 mg/ml), 2 μl (1.5 units) of DNA polymerase I (Klenow fragment) and finally 3 μl of 10 μCi/ μl of radiolabelled ($\alpha^{32}P$) dCTP (Amersham) was added. The labelling reaction proceeded at 37°C for 45 minutes.

The labelled probe was separated from unincorporated labelled nucleotide by passing it in TE through a column of Sephadex G 50 (Pharmacia) in a 1 ml syringe plugged with a piece of filter paper. 30 μl of 5 % (w/v) blue dextran and 0.5% (w/v) orange G was added to the probe as size markers. The blue dextran co-elutes with DNA and orange G with unincorporated nucleotides.

Hybridisation

Filters were pre-incubated in hybridisation buffer (0.5 % (w/v) skimmed milk powder, 1 % SDS, 4 x SSC) for between 15 minutes and 3 hours at 65°C. The probe was denatured by boiling for 5 - 10 minutes and then added to fresh hybridisation buffer. The filter was agitated gently overnight at 65 °C with the probe.

Washing filters

Filters were washed to remove unhybridised probe and so reduce background signal by shaking in wash buffer (2 x SSC, 0.5 % SDS) for 10 minutes - 1 hour at about 65°C (high stringency). The filter was then dried, wrapped in Saran wrap and exposed to X-ray film.

DNA Sequencing

Manual sequencing

Double stranded plasmid DNA was sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977). The ^{T7}Sequencing Mixes kit (Pharmacia) was used with T7 DNA polymerase (Pharmacia) to sequence mini-prep DNA. 10µl DNA (0.5µg/µl) was denatured by adding freshly prepared 2M NaOH at room temperature for 10 minutes. Spin columns were made by making a small hole in the bottom of a 500µl Eppendorf tube with a red hot needle and covering the bottom with glass beads. The tube was then half filled with Sepharose CL-6B. The column was placed inside a similarly perforated 1.5 ml Eppendorf tube. Both were then placed within a 15 ml Falcon tube (Greiner) and centrifuged at 750G for 2 minutes. The column was washed by adding water and spinning as before. The column was removed from the supporting 1.5 ml and 14 ml tubes and placed into a unperforated 1.5 ml tube. The denaturing reaction was added to the column and centrifuged once more to collect the DNA solution in the larger tube. 1µl of primer (5 µM) and 2 µl of annealing buffer (1M Tris.HCl pH 7.6, 100 mM $MgCl_2$ and 160 mM DTT) were added to the DNA and the mixture heated to 65°C and allowed to cool to 37°C so that annealing could take place. 3.0 µl of labelling mix-dATP (1.375 µM each dCTP, dGTP and dTTP; 333.5 mM NaCl), 2 µl enzyme dilution buffer (Pharmacia), 0.5 µl T7 DNA

polymerase (8-12 U/µl) and 0.5 µl [α^{35}-S]dATP (Amersham) was added to the annealed template and primer. After 5 minutes at room temperature, 4.5 µl of this labelling reaction was added to each termination reaction. Four termination reactions were used per template, each containing 2.5 µl of termination mix (93.5 µM relevant dNTP, 14 µM relevant ddNTP (dideoxy nucleoside 5' triphosphate), 840 µM each of the other three dNTPs, 40 mM Tris.HCl pH 7.6 and 50 mM NaCl). The reaction was incubated at 37°C for 10 minutes after which it was stopped by adding 5 µl of stop solution (0.3% each bromophenol blue and xylene cyanol FF; 10 mM EDTA pH 7.5, and 97.5% deionised formamide). Prior to electrophoresis on an acrylamide gel, the reactions were heated at 80°C for 3 minutes.

Two glass plates were cleaned and clipped together, separated by 0.5 mm spacers. Acrylamide gel mix (1 × TBE, 5% acrylamide/bisacrylamide [19/4] and 5M urea) was prepared. The lower space between the two plates was sealed using 20 ml of the gel mix polymerised using 75 µl each of a freshly prepared 25% (w/v) solution of ammonium persulphate and TEMED (N, N, N'-Tetramethylethylenediamine). The running gel was prepared by the addition of 70 µl each of a freshly prepared 25% (w/v) solution of ammonium persulphate and TEMED to 50 ml of gel mix. The running gel mix was poured between the glass plates. A *sharks tooth* comb was inserted into the top of the gel to generate wells into which 2.5 µl of the sequencing reaction was loaded. Electrophoresis at approximately 50 W in TBE for 2-8 hours followed. The gel was then fixed in 10% (v/v) methanol, 10% (v/v) glacial acetic acid for 15 minutes, transferred onto Whatman 3MM paper, covered with Saran wrap and dried under vacuum at 80°C for 30 minutes. Autoradiograhpy was performed using X-ray film at room temperature and exposed overnight. The resulting sequence data were analysed using the University of Wisconsin's GCG programmes (Devereaux *et al.*, 1984).

Automated Sequencing

Cycle sequencing using dye-labelled terminators was performed using the ABI Prism^{TM} Dye Terminator Cycle Sequencing Ready Reaction kits with AmpliTaq

DNA Polymerase, FS (Perkin-Elmer). Double stranded DNA was purified using a Qiagen plasmid mini prep. kit. The sequencing reaction comprised 500 ng DNA, 4 µl terminator ready reaction mix, 1.6 pmole primer in a total volume of 10 µl. The reaction was prepared in a thin walled 250 µl tube and placed in a Rapid cycler, thermal cycling carried out as follows; 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes, with maximum thermal ramp throughout. The extension products were stored at 4°C and purified to remove unincorporated terminators by ethanol precipitation of the DNA. To do this, the extension reaction was transferred to a 0.5 ml Eppendorf tubes containing 1 µl of 3M Sodium acetate, pH 4.6 and 25 µl of ethanol. These were mixed and incubated on ice for 10 minutes, centrifuged at 11500 *g* for 15 minutes, the ethanol solution removed, the pellet washed with 70% (v/) ethanol and vacuum dried.

Loading buffer was prepared by combining deionized formamide and 25 mM EDTA (pH8.0) containing 50 mg/ml Blue dextran in a ratio of 5 : 1 formamide to EDTA/Blue dextran. The sample was resuspended in the 6-9 µl loading buffer, heated for 2 minutes to denature and loaded onto an ABI Prism 377 sequencing gel (Perkin Elmer). Sequencing data was analysed using the GCG9 computer package.

Screening a cDNA library

A cDNA library in λgt10, made from young wild type inflorescences was a gift from R. Simon and E. S. Coen, John Innes Institute, Norwich, UK. The library was screened at a density of 10, 000 plaques per plate (9cm diameter).

Preparation of plating cells

For λgt10 in which inserts disrupt the *CI* gene, the host cells C600 *hfla*⁻ were used to select for recombinants. A single colony was cultured overnight at 37°C in 5 ml of L broth supplemented with 50 ml 1M $MgSO_4$, 50 ml 20 % (w/v) maltose and 7.5 ml of 10 mg/ml tetracycline. The cells were spun down, the medium was removed and the cells resuspended in 10 mM $MgSO_4$ to give an absorbance at 600 nm of 1.2 in a 1 cm light path.

Preparation of plates and top agar

LB agar plates were poured, allowed to set and then dried thoroughly in an 45°C oven. BBL top agar (0.1% Oxoid tryptone soya broth, 0.5% NaCl, 0.6% Difco agar) was melted in a microwave and then supplemented with 1ml 1M $MgSO_4$ and 1 ml 20% (w/v) Maltose per 100 ml agar. 3.5 ml of the top agar per plate was aliquotted into warmed test tubes held in a 47°C water bath.

Infection of cells

100 ml of the prepared plating cells were used per plate. Each aliquot of plating cells was infected with 1 µl of the library for 20 minutes at 37°C. The infected cells were added to the warm top agar and immediately poured onto the LB plates, ensuring that the plates were level. After the agar has set the plates were incubated overnight at 37°C.

Plaque lifts

The plates were chilled at 4°C for a few hours to harden the top agar. Nitro-cellulose filter discs (Hybond-C gridded membranes, 0.45 mm pore size, Amersham) were places on the agar. Pin holes were made through the surface of the disc into the agar, as orientation marks. After 5 minutes, the filter was transferred to denaturing solution, neutralising solution and 2 x SSC (as for Southern blots) for 5 minutes each. The filters were allowed to air dry for approximately 30 minutes, after which the DNA was fixed by baking under vacuum at 80°C for 45 minutes. The filters were hybridised in a circular plastic container as described for Southern blots, except that the filters were pre-hybridised for about 2 hours to remove any agar and bacterial debris.

Screening the library by hybridisation

The discs from the plating represented the first round screen. Following hybridisation, the discs were exposed to X-ray film and positive plaques were identified. The agar coresponding to the positive plaque was cored out using the wide

end of a yellow tip. The phage was eluted from this agar core in 1 ml of 10 mM $MgSO_4$. From a 1 x 10^{-5} dilution of this eluate, 1 ml was plated out with 100 ml of plating cells for the second round. This produced about 600 plaques per plate so that individual plaques could be identified and isolated as being pure positive phage from this round.

In situ Hybridisation

To investigate the expression pattern of *PHAN* and other genes, RNA probes were hybridised to sections of inflorescence and vegetative meristems. RNA probes were transcribed *in vitro* and labelled with digoxygenin-11-UTP. The labelled probes were detected by an immunochemical assay, using an anti-digoxygenin antibody conjugated to alkaline phosphatase. The antibody binds to digoxygenin and the alkaline phosphatase catalyses a colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT), so that signal is seen as a blue/brown precipitate. The method is based on those of Coen *et al* (1990) and Jackson (1992).

Fixation and wax embedding of material

Fresh meristems were fixed to preserve tissue morphology and to restrict RNA molecules to their position *in vivo*. The fixative acts by crosslinking RNA molecules to proteins and other macromolecules to form a three dimensional array.

Inflorescence meristems were harvested from the apices of mature *Antirrhinum* plants, while vegetative meristems were harvested from the apices of seedlings. The meristems were immediately fixed in freshly prepared 4% (v/w) formaldehyde. To make the fixative, paraformaldehyde was dissolved in phosphate buffered saline (PBS, 130 mM NaCl, 7 mM Na_2HPO4, 3 mM NaH_2PO4) which had been heated to 60°C and adjusted to pH 11 with NaOH. The solution was then chilled and the pH returned to pH 7 using H_2SO4. 0.1% (w/v) Triton-X-100 and 0.1% (w/v) Tween 20 were added to increase penetration of the fixative. Since the plant material had a cuticle, a vacuum was applied to aid penetration of the fixative into internal tissues. Formaldehyde vapour is volatile, so the fixative was replaced after the vacuum treatment before incubation overnight at 4°C.

Following the fixation stage, the tissue was washed in 0.85% (w/v) NaCl ("saline") to remove the formaldehyde and then dehydrated through an ethanol series. All formaldehyde stages were performed in the fume hood. The tissue was cleared with

Histoclear (Cellpath) and wax introduced by incubation in a mixture of histoclear and wax. Molten wax was then introduced. For 3 days the tissue was maintained at 58°C with wax being replaced with fresh every morning and evening. The details of these treatments are shown in Table A(i). The tissue was finally placed in moulds filled with molten wax. The moulds were floated on water to solidify the wax and stored at 4°C until sectioned.

Sectioning of wax embedded material

The wax block was trimmed into a trapezoid shape and mounted onto a metal plate with a little molten wax. The plate was secured onto a microtome (Reichet-Jung) so that the face of the block was parallel to the blade with the longer of the two parallel faces is at the bottom. Ribbons of sections 7 μm thick were cut and floated in sterile water onto Polysine (BDH) microscope slides. These slides were supplied ribonuclease free and coated in poly-L-lysine. The poly-L-lysine provided an even, positively charged surface to which plant tissue can adhere. The slides were placed on a 42°C hotplate overnight, to allow the ribbon to flatten and to dry onto the slides.

Tissue pre-hybridisation treatments

The treatments prior to hybridisation serve two main functions. Firstly, a brief protease digestion to permeabilise the tissue, thus increasing the accessibility of the target RNA in the tissue to the probe and antibody. Secondly, a treatment with acetic anhydride served to acetylate and neutralise positive charges so reducing non-specific binding of the probe.

Tissue mounted on slides were loaded into a stainless steel rack (Raymond Lamb) 24 at a time, and passed through the series of solutions outlined in Table A(ii). The initial Histoclear treatment cleared wax from the sections. The tissue was re-hydrated in an ethanol series. The limited protease digestion with Pronase was stopped by rinsing in 0.2% glycine. Any RNA dislodged during the protease treatment was refixed in the original location, in a 4% formaldehyde fixation stage. Following acetylation, the tissue was dehydrated

Table A(i). Treatment of tissue for wax embedding

Treatment	Time	Temperature
4% Formaldehyde	overnight	4°C
0.85% Saline	30 minutes	on ice
50%Ethanol/0.85% Saline	1.5 hours	on ice
70%Ethanol/0.85% Saline	1.5 hours	on ice
85%Ethanol/0.85% Saline	overnight	4°C
95%Ethanol/0.85% Saline	1.5 hours	4°C
100%Ethanol	1.5 hours	4°C
100%Ethanol	overnight	4°C
100%Ethanol	2 hours	Room Temperature
50%Ethanol/50% Histoclear	1 hour	Room Temperature
100% Histoclear	1 hour	Room Temperature
100% Histoclear	1 hour	Room Temperature
100% Histoclear	1 hour	Room Temperature
50%Histoclear/50% wax	overnight	58°C

Table A(ii). Tissue pre-hybridisation treatments

Treatment	Time
Histoclear	10 min
Histoclear	10 min
100% Ethanol / 0.85% Saline	1 min
100% Ethanol / 0.85% Saline	30 sec
95% Ethanol / 0.85% Saline	30 sec
85% Ethanol / 0.85% Saline	30 sec
50% Ethanol / 0.85% Saline	30 sec
30% Ethanol / 0.85% Saline	30 sec
0.85% Saline	2 min
PBS	2 min
Pronase (0.125mg/ml in 100mM Tris.HCl pH 7.5, 10mM EDTA)	10 min
Glycine (0.2% in PBS)	2 min
PBS	2 min
Formaldehyde (4% in PBS)	10 min
PBS	2 min
PBS	2 min
Acetic anhydride (0.5% v/v in 0.1M triethanolamine pH 8.0)	10 min
PBS 2	2 min
0.85% Saline	2 min

through an ethanol series and then rinsed in fresh ethanol. The slides could be stored over ethanol for up to 24 hours before hybridisation.

300 ml of each solution was sufficient to treat 2 racks of slides, except for the acetylation, where 600ml was used. Both Histoclear steps and the first ethanol step were carried out in glass troughs after which plastic boxes were used. Formaldehyde, acetic anhydride and Histoclear stages were performed in the fume hood. Acetic anhydride is unstable in water, so the slide rack was placed over a magnetic stirrer (supported on an empty slide rack) as fresh acetic anhydride was added. All steps were carried out at room temperature. Pronase (Sigma, type XIV) was made up as a 40 mg/ml stock, pre-digested at 37°C for 4 hours and stored at -20°C.

RNA labelling by transcription *in vitro*

The DNA to be transcribed was subcloned into the polylinker of pBluescript, adjacent to the T7 polymerase promoter. The DNA was subcloned separately in both orientations. 'Run off' transcripts were generated by linearising the plasmid at an appropriate site. An alternative was to amplify the insert and adjacent T7 promoter using M13 -40 and Reverse primers. The resultant PCR product could then be used as a template. Linearised templates were purified using phenol/chloroform extraction and ethanol precipitation.; PCR products were purified using a PCR purification column (Qiagen). The labelling reaction contained 10.5 µl water containing 1 µg of template DNA, 2.5 µl 10x T7 RNA polymerase transcription buffer, 1 ml RNAse inhibitor (20U/µl), 2.5 µl each of 5 mM ATP, GTP and CTP, 2.5 µl of 1 mM Dig-UTP and 1 µl of T7 bacteriophage RNA polymerase (20U/µl). All components were supplied by Boerhinger-Mannheim. The reaction proceeded at 37°C for 40 minutes and was stopped by the addition of 75 µl of MS (10 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl), 2 µl tRNA (100 mg/ml) as a carrier and 1µl of DNAse I (10 U/µl) to digest the DNA template. After 10 minutes at 37°C the RNA was precipitated with 100 µl 3.8 M ammonium acetate and 600 µl ethanol, pelleted, washed in 70% ethanol/1.5 M NaCl and dissolved in 50 µl of water. To hydrolyse the probes, an equal volume of carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3) was added and the reaction was heated to 60°C. The optimum length for probes is

approximately 150 bp. In order to hydrolyse different probes to the desired average length, the hydrolysis time was calculated using the following formula (Cox *et al* 1984):

$t = Li - Lf / K.Li.Lf$

Where t = time in minutes, Li = initial probe length in kb, Lf = final probe length, K = rate constant (0.11 kb/min).

To stop hydrolysis, 10 µl of 10% acetic acid, 12 µl of 3M sodium acetate and 312 µl of ethanol were added and the RNA again precipitated, washed and dissolved in 50 µl TE. The RNA probes were then stored at -80°C until use. To check the incorporation of the dig-UTP label, 0.5 µl of probe was spotted onto nylon filter, along with labelled (100 µg/ml) and unlabelled (200 µg/ml) control RNA (Boerhinger-Mannheim) and UV crosslinked. An abbreviated detection method was then performed. The filter was briefly wetted with buffer 1 (100 mM Tris.HCl pH 7.5, 150 mM NaCl with 0.3% (v/v) Triton-X-100), incubated in buffer 2 (0.5% (w/v) blocking reagent [Boerhinger-Mannheim] in buffer 1) for 30 minutes to block the filter, washed with buffer 1, incubated with buffer 1 containing anti-Dig antibody (1 : 5000 dilution of 0.75 U/ml stock), washed in buffer 1 and briefly in buffer 5 (100 mM Tris.HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2) to raise the pH. Buffers 1, 2, 5 and 6 were made up as for the immunological detection of digoxygenin labelled probes (see ahead). The filter was incubated in buffer 6 (buffer 5 with 0.34 mg/ml NBT and 0.175 mg/ml X-phosphate) for 10-15 minutes. The intensity of the purple/brown stains relative to the controls gave an estimate of the success of the *in vitro* transcription.

Hybridisation and washing

Riboprobes were used at a concentration of 0.1-0.3 ng/ml/kb probe length. For each slide, the final hybridisation mix was made up of 2 ml probe, 2 ml water, 4 ml formamide and 32 ml hybridisation buffer (50% formamide, 10% (w/v) dextran sulphate, 10 mM Tris.HCl, 300 mM NaCl, 10 mM $NaPO_4$ pH 6.8, 5 mM EDTA, 1 x Denhart's [0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA fraction V] and 1 mg/ml tRNA. The final hybridisation mix therefore consisted of 1 part probe, (5 x

concentrated in 50 % formamide) and 4 parts hybridisation buffer. The probe, water and formamide mixture was heated to 80°C for 2 minutes and cooled on ice prior to adding the hybridisation buffer. The hybridisation mix was then applied onto the slide and distributed evenly by placing a glass coverslip onto it. The slides were then incubated overnight at 50°C, elevated above paper towels soaked in wash buffer (50% formamide and 2 x SSC) in sealed boxes.

Following the hybridisation step, coverslips were removed by placing the slides in racks and immersing them in wash buffer at 50°C for 30 minutes. The slides were then washed twice in fresh wash buffer for an hour each and rinsed twice in NTE (500 mM NaCl, 10 mM Tris.HCl pH 7.5, 1 mM EDTA) for 5 minutes each. They were then incubated in RNAse A (20 ug/ml in NTE) at 37°C for 30 minutes to remove single-stranded, non-specifically bound probe. Two 5 minute rinses in NTE were then carried out at room temperature followed by a final incubation at 50°C in wash buffer for an hour.

Immunological detection of digoxygenin labelled probes

The slides in the rack were rinsed in buffer 1 (100 mM Tris.HCl pH 7.5, 150 mM NaCl with 0.3% (v/v) Triton-X-100) for 5 minutes, Buffer 2 (0.5% (w/v) blocking reagent [Boerhinger-Mannheim] in buffer 1) for an hour and buffer 3 (1% (w/v) BSA [Sigma] in buffer 1) for 30 minutes. This was followed by an hour long incubation with the Anti-digoxygenin-AP antibody (Boehringer-Mannheim) at a concentration of 1 : 3000 in buffer 3. For the antibody incubation stage, the slides were transferred to square petri dishes to minimise the volume of buffer and so minimise the amount of antibody necessary. The slides were then returned to the rack and washed 4 times for 20 minutes each with buffer 1 There was a 5 minute wash with buffer 1 which did not contain triton, followed by another brief wash in buffer 5 (100 mM Tris.HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2). The slides were again transferred to square petri dishes and incubated in buffer 6 (buffer 5 with 0.34 mg/ml NBT and 0.175 mg/ml X-phosphate) for 2 days in the dark. The enzyme reaction was stopped by washing in water. A two minute wash in 70% (v/v) ethanol removed background. Counter-staining of the cell walls was carried out in 0.1%(w/v) Calcafluor White (Sigma) for 2

minutes. The sections were rinsed briefly in distilled water and allowed to air dry, before mounting with Entellan (Merk). The sections were viewed and photographed using a Polyvar microscope (Riechert-Jung) under a mixture of UV and brightfield illumination.

Results

The *PHANTASTICA* gene of *Antirrhinum majus*

Introduction : Isolation of the *PHAN* locus

The *PHANTASTICA* gene encodes a MYB-related transcription factor

phan mutant alleles

PHAN is Expressed in all Lateral Organs

The *phan* Mutant Phenotype is Temperature Sensitive

Genetic Interactions at the Meristem

Introduction : Isolation of the *phan* locus

Mutants of the *phan* locus showed a reduction in the dorsoventrality of leaves, bracts and petal lobes (Waites and Hudson, 1995). While all mutants showed the same range of vegetative phenotypes, each shows a characteristic reduction of the petal lobes. To allow molecular characterisation of the *PHAN* gene, the locus had been isolated following a targeted mutagenesis screen by Richard Waites (Waites *et al.*, 1998). Stable *phan* mutant lines were crossed to transposon active wild type lines and 18000 F1 progeny screened for a new *phan* mutation. A new mutant allele, *phan*-552, had been identified which showed the genetic instability characteristic of transposon induced mutations and an insertion of the transposon Tam 4 found to be responsible. This transposon was present in the *phan*-552 mutant, but not in wild type siblings or revertants obtained by back-crossing *phan*-552/*phan*-249G with its mutant parent *phan*-249G/*phan*-249G. Tam 4 was identified in a 6 kb *Bgl* II fragment. The *Bgl* II fragment was isolated and 1.6 kb of DNA flanking the right hand end of the transposon was used as a probe to detect a polymorphic band that was present in the other mutant alleles but absent in their wild type progenitors, providing strong evidence that the flank contained at least part of the *PHAN* gene. To test whether this flanking region contained the *PHAN* transcribed region, it was used as a probe in Northern hybridisations. It detected a transcript of ~1.4 kb only in mRNA from wild type shoot tips and not from *phan*-249G mutant shoot tips. Therefore the flank was likely to contain at least part of the transcribed region. To allow the transcription product to be analysed, an inflorescence cDNA library was screened using this right hand flank as a probe. Twenty positives resulted, all with insertions about 1.4 kb in size, suggesting that they were near full length clones.

The *Eco* RI-*Bgl* II fragment to the right of the Tam 4 insertion was sequenced and was shown to contain an open reading frame. Gillian Renee sequenced the cDNA. Comparison of the sequence of the cDNA with the genomic sequence showed that this genomic region contained the entire PHAN protein coding region and was free of any introns. However, 5' RACE (rapid amplification of cDNA ends) analysis, performed by Ian Oliver, revealed the presence of an intron (size unknown) in the untranslated leader sequence, into which Tam 4 had inserted in *phan*-552. There were two potential splice donor sites 50 bp upstream of the start of translation and 132 or 139 bp downstream of the major start of transcription with the acceptor site at 140 bp downstream of the start of transcription (Figure 9). There were two possible sites for the start of transcription, 23 bp apart, as indicated by two different size classes of RACE clones. It was assumed that the more upstream position was the major start site, since RACE clones of this size were more frequent.

Translation was assumed to started at position 188 with an ATG codon and to proceed till position 1258 where it terminated on a TAA codon, such that the last amino acid residue was arginine. This open reading frame showed the potential to encode a 42.4 kD PHAN protein of 356 amino acids. PHAN had two tandem imperfect repeats of 56 and 51 amino acids at the N-terminus. These motifs were found to be conserved between the MYB family of transcription factors (Figure 10). The MYB DNA binding domain consists of three or usually two such repeats in vertebrates and plants, respectively. Structural analysis of the vertebrate MYB protein had revealed that each repeat folds into a helix-turn-helix motif with three helices in each repeat. Regularly spaced tryptophan residues (3 in the first repeat, R2 and 2 in the second repeat, R3) are also conserved between plant MYBs (Figure 10). These residues are thought to play a role in maintaining the hydrophobic core of the DNA binding domain. Unlike other plant MYBs, PHAN showed little conservation in the C-terminal region of the second repeat (residues 93-102), had only a single

```
>                                                    >
TTTTTACCCGCGTGACAACTTTTTACTCTCTTTTAATCAGAGTTTCTTGTTCTCAGCCTT    60
AGAGAAAAAAAAAGGGCTGTTCTTTCATGGTACAGATAATTATGGGCTTTGGAGAAAACTT   120
TGTTTCTTGAATgtcagtATAAATATGAAAATTTTGGGGCAATGGGCAAAGGTTTTTGCG   180
AGTGGAAATGAAGGAAAGGCAACGATGGAGACCTGAAGAAGACGCCTTGTTGCGTGCTTA   240
        M  K  E  R  Q  R  W  R  P  E  E  D  A  L  L  R  A  Y     18

TGTAAAAGAATACGGCCCCAGAGATTGGCACCTAGTGACACAACGCATGAACAAACCTCT   300
 V  K  E  Y  G  P  R  D  W  H  L  V  T  Q  R  M  N  K  P  L     38

CAACCGGGACGCGAAATCCTGTTTAGAAAGGTGGAAAAACTATTTAAAACCCGGAATCAA   360
 N  R  D  A  K  S  C  L  E  R  W  K  N  Y  L  K  P  G  I  K     58

GAAAGAATCCCTTACACAAGAGGAGCAGATTCTCGTTATTAATCTACAAGCGAAACACGG   420
 K  E  S  L  T  Q  E  E  Q  I  L  V  I  N  L  Q  A  K  H  G     78

CAACAAGTGGAAAAAGATCGCTGCTGAAGTTCCAGGACGAACTGCCAAAAGACTTGGTAA   480
 N  K  W  K  K  I  A  A  E  V  P  G  R  T  A  K  R  L  G  K     98

GTGGTGGGAAGTTTTTAAAGAGAAGAAGCAACGCGAAGAAAAGGACAACAAGAAGATTAC   540
 W  W  E  V  F  K  E  K  K  Q  R  E  E  K  D  N  K  K  I  T    118

CGAACCGATTGAAGAAGGAAAATACGATCGTATATTGGAGACGTTTGCAGAGAAGATAGT   600
 E  P  I  E  E  G  K  Y  D  R  I  L  E  T  F  A  E  K  I  V    138

AAAAGAGCGAGTCGTTTCAAGAATTATTACAATGCCGCCAACTTCAAATAGTGGATTTCT   660
 K  E  R  V  V  S  R  I  I  T  M  P  P  T  S  N  S  G  F  L    158

TCAAAACGATCCGTCTCCGCATTCTGCACAATCGGTGTTACCCCCATGGCTAGCTAGTTC   720
 Q  N  D  P  S  P  H  S  A  Q  S  V  L  P  P  W  L  A  S  S    178

TAGCATGACAACAACCATTAGGCCACAATCCCCGTCCGTGACTTTATCCCTCTCGCCCTC   780
 S  M  T  T  T  I  R  P  Q  S  P  S  V  T  L  S  L  S  P  S    198

TGTAGTGCCTCCTGCCCCAGCAATCCCCTGGCTACACCCTGATAACACCACTCATGGTCC   840
 V  V  P  P  A  P  A  I  P  W  L  H  P  D  N  T  T  H  G  P    218

GAGCAATTTGTCGTCTCTTGGGGTAGTTGCGCCTTTTATGGGAGAAAACCATATAGTTCC   900
 S  N  L  S  S  L  G  V  V  A  P  F  M  G  E  N  H  I  V  P    238

TGAACTTTTAGAGTGCTGTCGGGAATTGGAAGAAGGGCAGCGTGCATGGGCGGCGCACAG   960
 E  L  L  E  C  C  R  E  L  E  E  G  Q  R  A  W  A  A  H  R    258

AAAAGAAGCAGCTTGGAGACTAAAAAGGGTAGAACTGCAGTTGGAATCGGAGAAAGCATG  1020
 K  E  A  A  W  R  L  K  R  V  E  L  Q  L  E  S  E  K  A  C    278

TCGAAGGAGAGAGAAAATGGAGGAGATTGAAGCGAAAATGAAAGCTCTTAGAGAAGAACA  1080
 R  R  R  E  K  M  E  E  I  E  A  K  M  K  A  L  R  E  E  Q    298

GAAGGCTAGTCTCGATCGGATCGAAGCAGAGTACAGGGAACAATTAGCCGGATTGAGAAG  1140
 K  A  S  L  D  R  I  E  A  E  Y  R  E  Q  L  A  G  L  R  R    318
                                                    S  T

AGAAGCGGAAGTGAAAGAACAGAAGCTGGCTGAGCAATGGGCGGCTAAGCACTTGCGTCT  1200
 E  A  E  V  K  E  Q  K  L  A  E  Q  W  A  A  K  H  L  R  L    338
 T  T  K  N  P  Y  W  D  T  E  M  C  P  K  I  D  L  L  Q  H

AACTAAGTTTCTCGAGCAGACGGGGTATAGGTCGATTGCTGGTGAGCTGAATGGTCGATA  1260
 T  K  F  L  E  Q  T  G  Y  R  S  I  A  G  E  L  N  G  R      357
 L  L  Y  G  T  L  A  I  S  V  A

AAGGCGTTCTGCTTTGTTGTTATGTTGGTCTACTTCTTCTATAACAGATATATGGTATTT  1320
GCTTGAGTTAATTTCTTAGGTCTTGAATCAACCTTGGCTAGATTGTTTTTGCTTTTCATT  1380
ATAGATCTGTGTGATCTGTGTGATCTTGATTTGGATTTTGGTACACCTTTAAAGGTTGGT  1440
TTATGTTATTGAGAATGTGATGAGGAACTATGGTTAAAATAGAGAATAAAAAAAAAA     1497
```

Figure 9. The amino acid sequence of PHAN is shown below its nucleotide sequence. The 5' end obtained by RACE is assumed to represent the longest transcript. A second transcription initiation site is indicated by an arrow at position 25. Splicing of an intron in the 5'UTR can occur at two donor sites separated by the sequence in lower case. The two MYB repeats are underlined and the novel sequence resulting from the insertion of *Tam2* in *phan*-249G is shown in green italics.

amino acid (methionine) upstream of the first repeat (Figure 9) and contained two or three extra amino acids in the first repeat (residues 36-38, Figure 10).

In common with other MYB proteins, the C-terminus was weakly acidic and had the potential to form an alpha helical structure which may be involved in transcriptional activation (Paz-Arez, *et al.* 1987). The region downstream of the PHAN DNA binding domain typically showed little conservation with previously isolated MYB genes. Following the isolation of *PHAN*, other *PHAN*-like genes, showing homology in the C-terminal region, have been identified in both monocotyledons and dicotyledons (J. Langdale, R. Waites, A. Hudson, Neil McHale, pers. comm). The only other similarity was to the product of a gene of unknown function, induced upon root-knot nematode infection in tomato (Bird and Wilson, 1994). Therefore, it is probable that PHAN is a member of an ancient subfamily of the MYB family of transcription factors that was present prior to the divergence of monotyledons and dicotyledons (Figure 11).

***phan* mutant alleles**

Four *phan* mutations had been identified (Waites and Hudson, 1995), which conditioned a similar vegetative phenotype. However, floral morphology was affected to different degrees, in a manner characteristic for each mutation, with *phan*-552 having the least severe floral phenotype and *phan*-250G having the most extreme effect on floral morphology (Waites and Hudson, 1995) (Figure12). This suggested that the weaker mutations retained some activity, that is, they were hypomorphs.

The petal lobes of *phan*-250G were reduced to needle made up entirely of ventral petal cell type. Petal lobes of *phan*-249G and *phan*-552 are reduced and have needles on their ventral surfaces. Patches of ventral petal epidermis are seen on the dorsal petal surface. As with the broader leaves, there is ectopic proliferation at the boundaries of these patches in a novel axis. *phan*-607 and *phan*-709 have similar, but less severely affected petal lobes. Therefore, Waites and Hudson (1995)

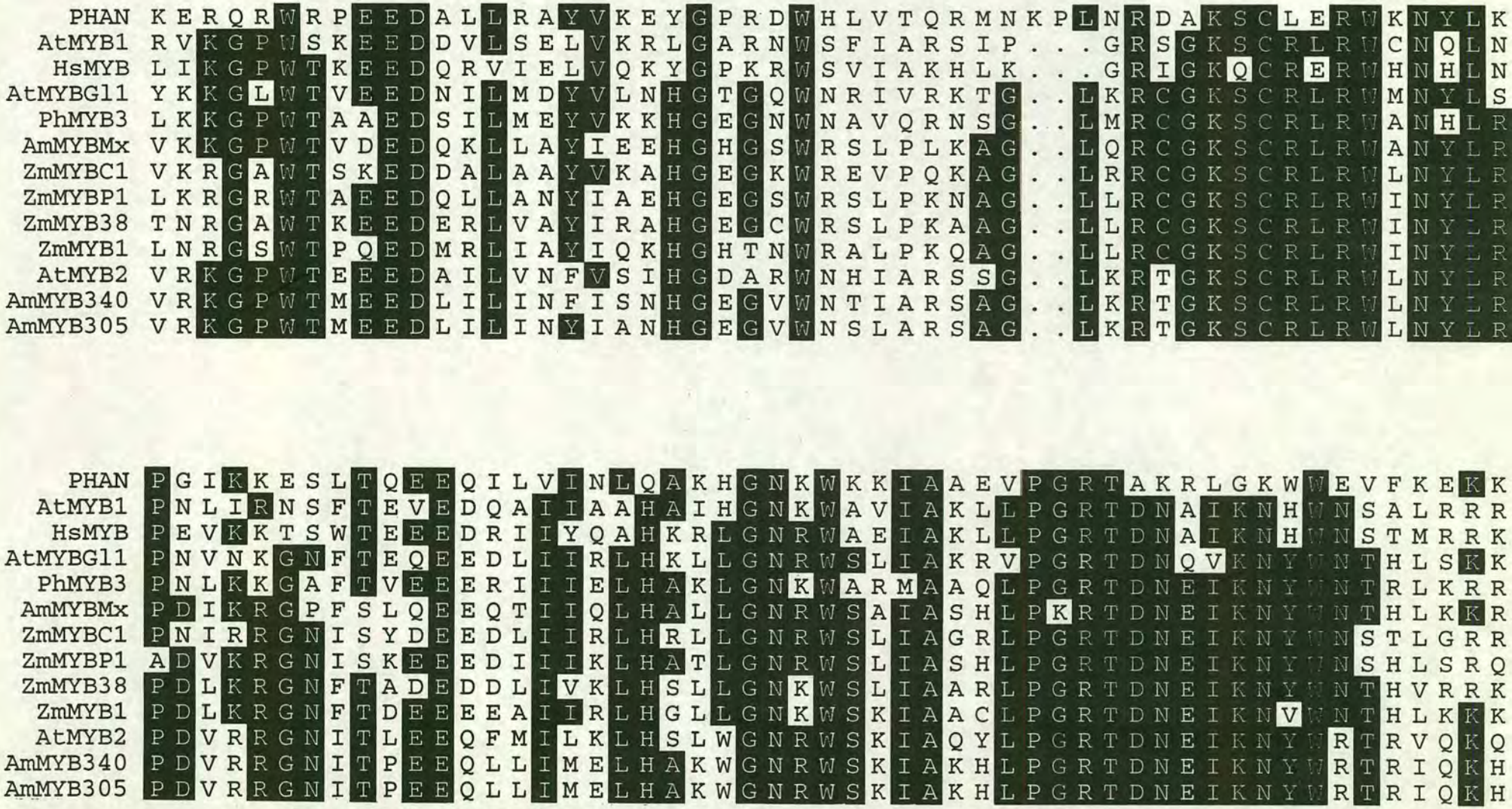


Figure 10. The *PHAN* MYB domain aligned to other MYB transcription factors which have defined functions and/or demonstrated DNA binding activities. Residues which are identical in the majority of these are boxed. The nomenclature for MYB proteins is that of Martin and Paz Ares (1997).

```
      1                                                          50
phan  MKERQRWRPE EDALLRAYVK EYGPRDWHLV TQRMNKPLNR DAKSCLERWK
 tob  MRERQRWRSE EDALLRAYVK QYGPKEWHLV SQRMNTALNR DAKSCLERWK
arab  MKERQRWSGE EDALLRAYVR QFGPREWHLV SERMNKPLNR DAKSCLERWK

      51                                                        100
phan  NYLKPGIKKE SLTQEEQILV INLQAKHGNK WKKIAAEVPG RTAKRLGKWW
 tob  NYLKPGIKKG SLTQEEQRLV IHLQAKHGNK WKKIAAEVPG RTAKRLGKWW
arab  NYLKPGIKKG SLTEEEQRLV IRLQEKHGNK WKKIAAEVPG RTAKRLGKWW

      101                                                       150
phan  EVFKEKKQRE EKDNKKITEP IEEGKYDRIL ETFAEKIVKE RV......VS
 tob  EVFKEKQHRE QKENNKVVDP VDEGKYDHIL ETFAEKIVKE RS......VP
arab  EVFKEKQQRE EKESNKRVEP IDESKYDRIL ESFAEKLVKE RSNVVPAAAA

      151                                                       200
phan  RIITMPPTSN SGFLQNDPSP HSAQSVLPPW LASSSMTTTI RPQSPSVTLS
 tob  GLLM..ATSN GGFLHADAPA PSPQTLLPPW LSNSTATSTV RSPSPSVTLS
arab  AATVVMANSN GGFLHSEQQV QPPNPVIPPW LATSNNGNNV VARPPSVTLT

      201                                                       250
phan  LSPSVVP... .PAPAIPWLH PD...NTTHG PSNLSSL..G VVAPFMGENH
 tob  LSPSTVPPTP TPTPGIPWLQ TDR..GPENA PLILSSFPHH GVAPPCGENP
arab  LSPSTV.AAA APQPPIPWLQ QQQPERAHNG PGGLVLGSMM PSCIGSIESV

      251                                                       300
phan  IVPELLECCR ELEEGQRAWA AHRKEAAWRL KRVELQLESE KACRRREKME
 tob  FVTELVECCK ELDEGHRAWA AHKKEAAWRL RRVELQLESE KICKVREKME
arab  FLSELVECCL KLEERHRTWA DHKKEAAWRL KRLELQLESE KTCLQREKME

      301                                                       350
phan  EIEAKMKALR EEQKASLDRI EAEYREQLAG LRREAEVKEQ KLAEQWAAKH
 tob  EIEAKMKALR EEQKATLDRI EAEYKEQLAG LRRDAEAKEQ KLAEQWASKH
arab  EIEAKMKALR EEQKNAMEKI EGEYREQLVG LRRDAEAKDQ KLADQWTSRH

      351                  384
phan  LRLTKFLE.Q TGYRSIAGEL NGR~
 tob  LRLSKFLE.Q MGCQSRLAEP NGGR
arab  IRLTKFLEQQ MGCRLDRP~~ ~~~~
```

Figure 11. *PHAN* belongs to a sub-family of the MYB transcription factors. The amino acid sequence of *PHAN*-like genes from tobacco (tob) and *Arabidopsis* (arab) which are similar to *PHAN* in the MYB domain and the C-terminal region. Identical residues in the majority are shown in green.

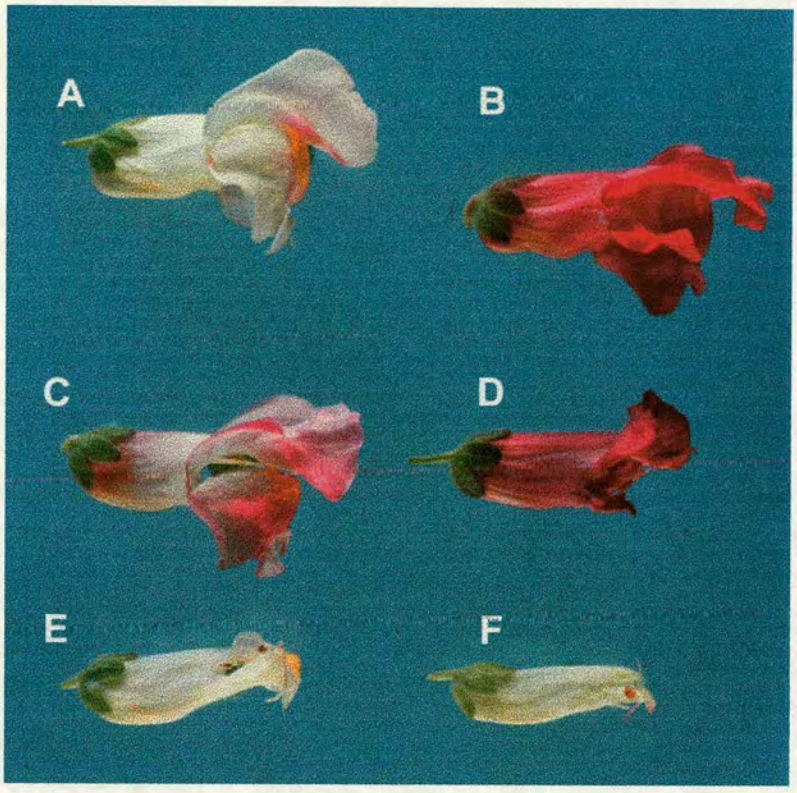


Figure 12. *phan* floral morphology. The wild type flower (A, B) is made up of five united petals to make the corolla tube, which then separate to give five petal lobes. *phan*-552 (C), *phan*-607 (D) and *phan*-249G (E) flowers have progressively greater reductions in lobe tissue. The petal lobes of *phan*-250G (F) have been reduced to needles.

proposed that the model explaining leaf development was also applicable for petal development.

In order to relate the structure of the *phan* mutant alleles to their characteristic phenotype, the precise nature of each mutation needed to be determined. Digestion of genomic DNA from each mutant doubly with *Bgl* II and a variety of restriction enzymes, followed by Southern analysis using the 1.6 kb *Bgl* II fragment as a probe, showed that all the *phan* mutant alleles contained insertions within the *Bgl* II fragment (Figures 13 and 14). The genomic map in Figure 14 is not to scale to allow clear representation of insertions within in the smaller *Bgl* II/*Eco* RI and smaller *Bgl* II/*Eco* RV fragments. The Southern blot showed weakly hybridising bands from a homologous sequence, for example, the approximately 700 bp band in the *Eco* RI digestion of wild type DNA (Figure 13). Partial digestion of the DNA by *Pst* I and *Eco* RV has occurred in the case of *phan*-249G and *phan*-250G, for example, the remaining 4 kb *Bgl* .II band seen in the *Pst* I digestion of *phan*-249G DNA.

In the case of *phan*-249G, Southern analysis revealed polymorphisms within the larger *Bgl* II / *Eco* RI, *Bgl* II / *Eco* RV and smaller *Bgl* II / *Pst* I fragments. These were consistent with the presence of a transposon of about 5 kb downstream of both the *Eco* RV site and the *Pst* I site. The size of the insertion and the introduction of novel restriction sites (*Pst* I and *Eco* RI) suggested that the transposon responsible in this case was Tam 2. This region was cloned from size fractionated DNA of *phan*-249G homozygotes using a *PHAN* probe. Sequence analysis confirmed that *phan*-249G carried an insertion of Tam 2 within the coding region so that the allele had the potential to encode a novel protein in which the C-terminal 41 amino acids of PHAN were replaced by 34 residues encoded for by Tam 2 (Figures 9 and 15). An additional two nucleotides (TC) at the upstream end of Tam 2, at position 1135 bp, was suggestive of an aberrant insertion event at this end of the transposon. It was assumed that the protein that results is partially functional since *phan*-249G plants show a mutant phenotype of intermediate severity.

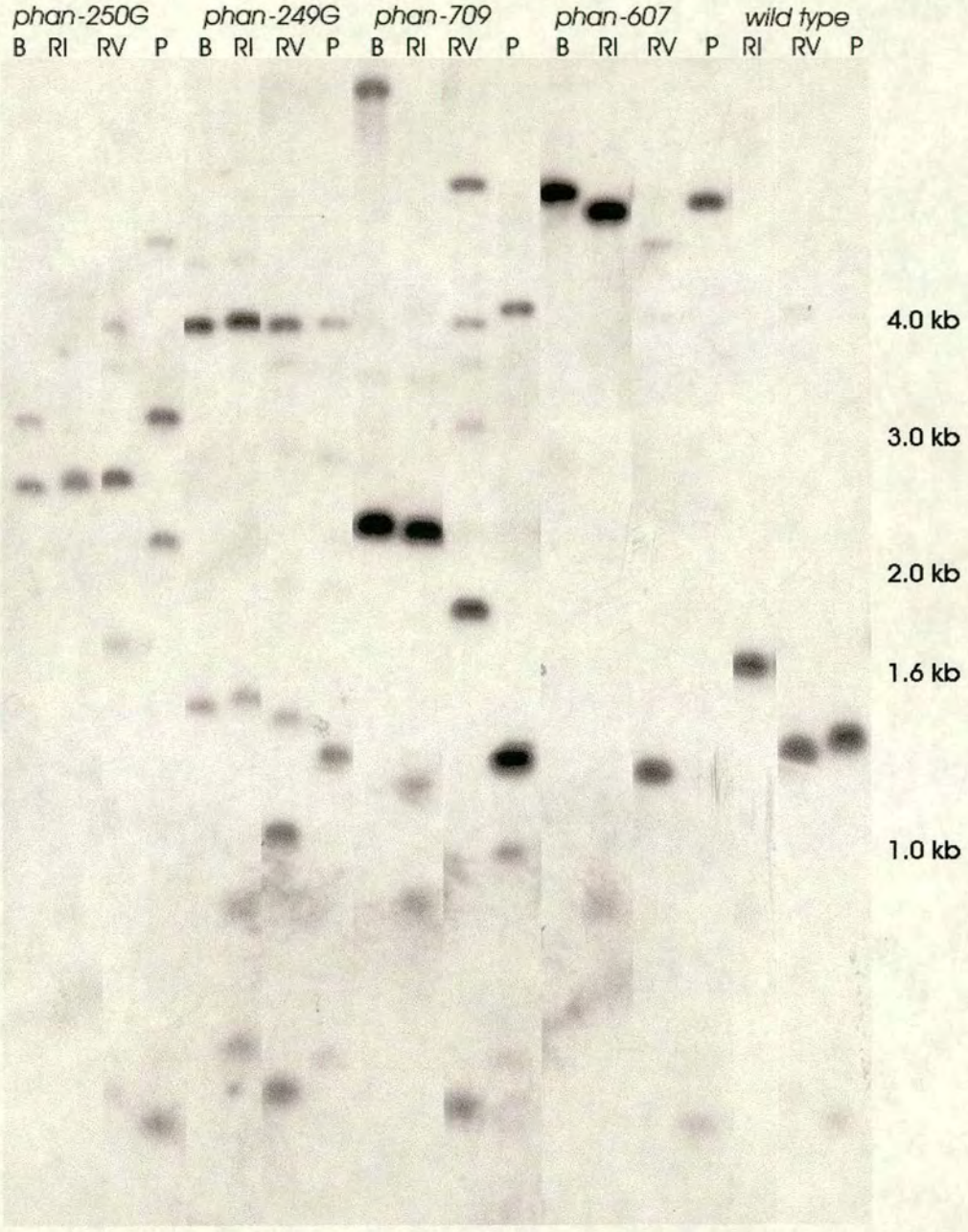


Figure 13. Southern hybridisation of wild type and *phan* mutant genomic DNA, digested doubly with *Bgl* II and a variety of enzymes (B = *Bgl* II, RI = *Eco* RI, RV = *Eco* RV and P = *Pst* I). The 1.6 kb region flanking the Tam4 insertion from *phan*-552 was used as a probe.

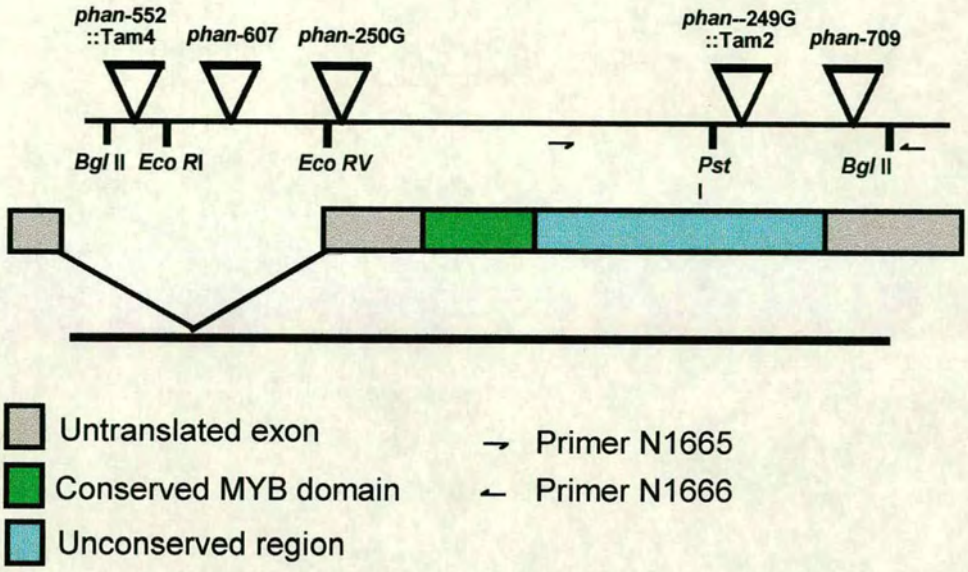
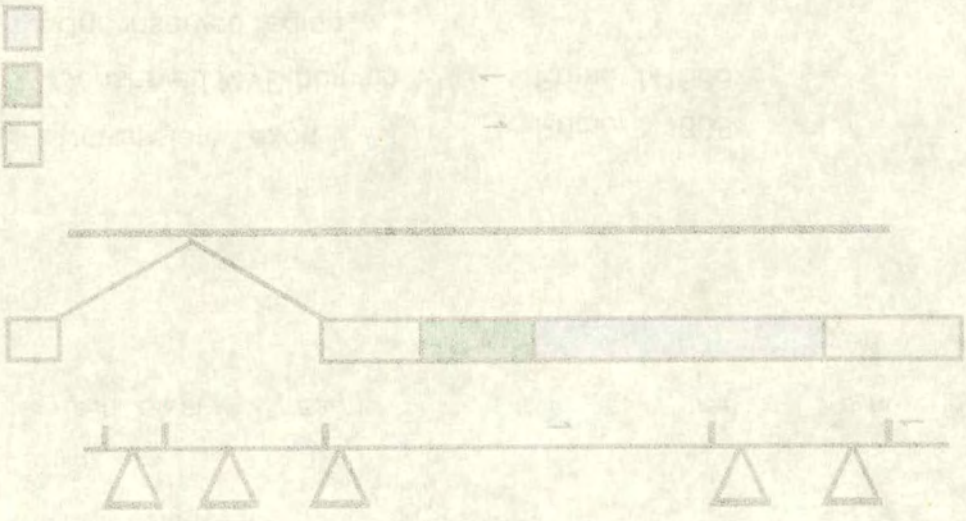


Figure 14. Map of the *PHAN* genomic region showing sites of transposon insertion, indicated by triangles (not to scale). Filled boxes denote exons (0.015 cm represents 1 amino acid residue). Primers N1665 and N1666 were used to amplify a region of the unconserved domain for use as a template for a probe in expression studies.



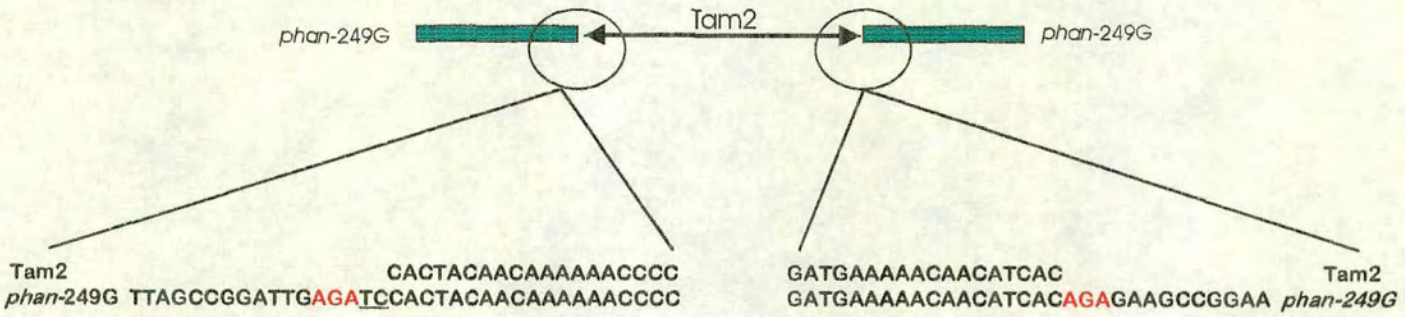


Figure 15. Insertion of Tam 2 into the coding region of *phan*-249G. The upstream and downstream ends of Tam2 are shown to match up with the genomic sequence of *phan*-249G. The insertion was impresise with the addition of nucleotides (TC) at the upstream insertion site. The three nucleotide target duplication is shown in red.

Polymorphisms were detected in *phan*-607 indicating an insertion downstream of the *Eco* RI site but upstream of both the *Eco* RV and *Pst* I sites, consistent with a transposon insertion within the intron. *PHAN* transcripts were detected in *phan*-607 mutant tissues at reduced abundance (see expression studies, discussed later) suggesting that the transposon sequence could be spliced out to allow a functional transcript to be made. Insertions within introns are known to have a disruptive effect upon splicing giving a less abundant product. This would account for the *phan* mutant phenotype conditioned by 607 plants. So, both *phan*-607 and *phan*-552 have insertions within the intron to give similar weak mutant phenotypes. However, *phan*-552 appeared slightly weaker than *phan*-607. Perhaps in the case of *phan*-552, splicing was less affected by the insertion than in *phan*-607 due a positional effect, so that abundance of the product was not as greatly affected in *phan*-552.

The most severe allele, *phan* 250G carried polymorphisms in the *Eco* RI fragment and in the larger Eco RV and *Pst* I fragments indicating an insertion between the *Eco* RV and *Pst* I sites. To further characterise the site of insertion, PCR was carried out using one primer within the intron (5' CTCCCACATGATATCA 3'), pointing downstream and another primer (5' CGATTGTGCAGAATGCGG 3') pointing upstream from the *Pst* I site. These primers did not amplify DNA from *phan*-250G genomic DNA, but were able to amplify a fragment from wild type genomic DNA. However when the upstream primer was changed for one that started at the ATG start of translation (5' GGCATATGAAGGAAAGGCAACGA 3'), a product was amplified from both *phan*-250 and wild type genomic DNA. This suggested that *phan*-250G had an insertion upstream of the start of translation and downstream of the *Eco* RV site. This insertion therefore had the potential to terminate transcription upstream of the PHAN protein coding region. This would explain the extreme mutant phenotype of *phan*-250G. Supporting evidence came from expression studies, discussed later, in which no transcript was detected in *phan*-250G while transcript was detected at lower levels in *phan*-607. The size and restriction map of the insertion did not correspond to that of a known transposon.

phan-250G has been shown to be a germinally stable mutant in different genetic backgrounds, including Garteslaben and JI. 75. Since no transposons that are either

germinally or somatically stable, it is likely that *phan*-250G is also somatically stable. It was said to be unstable by Stubbe (1934), though no figures were published. It is likely that this proposed instability was a result of the mosaic phenotype which looks like it results from somatic reversion events. However, it is more probable that the mosaic phenotype is due to other factors, which at early stages of vegetative development, can perform the same role as *PHAN* in most parts of the leaf primordium.

phan-torros was supplied by Frederic Cremer at the Max Planck Institut für Züchtungsforschung, Köln, Germany. This mutant had a similar vegetative phenotype to other *phan* mutants. In the flowers, there was a characteristic reduction of the petal lobes, which were split, often for the length of the corolla tube (Figure 16). The effect along the length of the petal and the corolla tube may be due to petal/corolla mutations in that genetic background. Crosses into a different genetic background to verify that the effect on the petal and corolla is not related to *phan* were not made. When *phan*-torros homozygotes were selfed, 5% of the progeny were wild type suggesting that the mutation was unstable. To test allelism, *phan*-torros and *phan*-250G homozygotes were crossed. All of the 20 resultant seedlings had *phan* mutant phenotypes, suggesting that *phan*-torros and *phan*-250G were allelic. No revertants were seen, possibly due to the small number of seedlings observed. Selfing these F1 plants resulted in 17.6% of the progeny having a wild type phenotype. These wild type individuals were assumed to have inherited a revertant *PHAN* wild type allele derived from *phan*-torros because *phan*-250G had previously been shown to be stable, even in different genetic backgrounds . The rate of reversion appears to be greater than when *phan*-torros is selfed, probably due to the small sample size. When a *phan*-torros revertant was selfed, the progeny segregated in a 3:1 ratio of wild type to mutant phenotypes, supporting this view. The reversion frequencies need to be verified by increasing the sample size from 20 plants. Thus, since *phan*-torros seemed to be an unstable mutant allele of *PHAN*, it was likely to have been caused by the insertion of a transposon. Restriction and Southern analysis did not reveal any polymorphisms within the *Bgl* II fragment so that any insertion lay outside this region. It is likely that the insertion was either in the promoter, the intron upstream of the *Bgl*

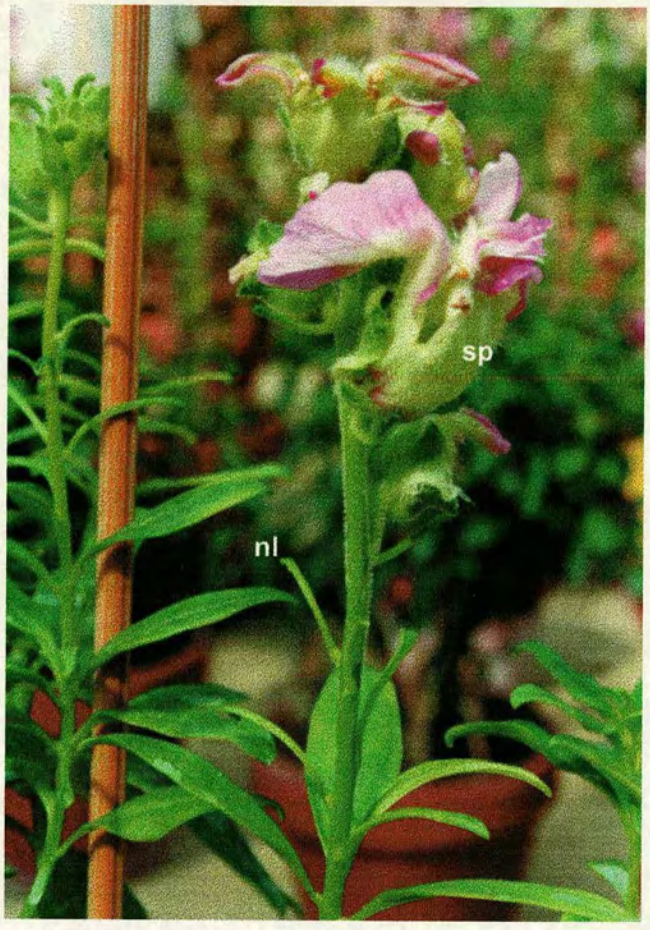


Figure 16. The *phan*-torros mutant. The vegetative phenotype shows needle-like leaves (nl). The characteristic floral phenotype shows reduced petal lobes which are often split (sp).

II site, or in the upstream exon, the position of which has yet to be determined. Although the results are generally consistent with *torros* being an allele of *phan*, there are other, less likely explanations. One of these is non-allelic-non-complementation, in which the trans-heterozygote has a mutant phenotype. If this were the case, the self of the F1 should have given 31.25% wild type plants. This analysis is complicated by the fact that *torros* is an unstable allele and reversion can account for wild type progeny. The other possibility that *torros* is a dominant allele appears unlikely because the progeny that result from crossing *torros* to wild type were only wild type.

A novel mutant allele, *phan*-709 was obtained during this work and shown to be allelic to *phan*. The vegetative phenotype was the same as for other mutants and the floral phenotype was similar to that of *phan*-607. An insertion was found in *phan*-709 within the *Bgl* II fragment, downstream of the *Eco* RI, *Eco* RV and *Pst* I. Therefore, the insertion is likely to be within the PHAN protein coding region or in the downstream untranslated region.

PHAN is Expressed in Lateral Organ Initials

The *phan* mutant phenotype suggested that *PHAN* plays a role in determining dorsoventrality in leaves, bracts and petal lobes. Waites and Hudson's model (1995) predicts that PHAN acts as part of a dorsalising function expressed at an early stage to determine dorsal cell identity. It had previously been found by Richard Waites that PHAN RNA was not detected in Northern blots, which agreed with the low frequency of clones in an inflorescence cDNA library (Richard Waites, pers. comm.).

To further characterise the role that *PHAN* plays in *Antirrhinum* development, the expression pattern of *PHAN* was investigated using *in situ* hybridisation, since it is more sensitive than Northern analysis. 700 bp of the unconserved region downstream of the MYB domain between primers N1665 (5'-ATGGCTAGCTAGTTCTAG-3')and N1666 (5'-CAAATCAAGATCACACAG-3') (Figure 14) was subcloned into the *Eco* RV site of pBluescript (KS and SK) and used for *in vitro* transcription of the sense and antisense strands of PHAN RNA from the T7 promoter. The RNA was labelled using digoxygenin-11-UTP (see Materials and Methods).

The expression pattern in vegetative apical meristems was investigated. Leaf primordia emerge in pairs from the opposite sides of the meristem, at approximately 90° to the previous pair of primordia (decussate phyllotaxy). As a pair of leaf primordia emerge from the flanks of the meristem as bulges, it is termed P_1, the previous pair being termed P_2. Prior to emergence, when leaf initials are morphologically indistinguishable from the meristem, they are known as P_0. Initial cells at earlier stages in the meristem are termed I_1, I_2 and so on. In wild type vegetative apices, no signal was seen using the control sense strand as a probe. When the antisense strand was labelled and hybridised with serial sections of wild type vegetative meristem, *PHAN* mRNA was detected at a very early stage, marking leaf initials before they became morphologically distinct from the rest of the meristem. As leaf primordia emerge at P_1, *PHAN* mRNA levels were maintained but became much lower by P_2 onwards (Figure 17B, 17C). Therefore, *PHAN* is expressed at a very

early stage, in accordance with phenotypic differences between wild type and *phan* mutants which can first be seen as primordia become distinct from the meristem (Waites and Hudson, 1995). However, PHAN expression is strong at P_0, suggesting that *PHAN* might act earlier than was suggested by the phenotype. The Waites and Hudson model suggested that *PHAN*'s expression pattern might be dorsally restricted. However, *PHAN* is expressed in all cells of the primordium. In contrast to the wild type apices, vegetative apices from *phan*-250G showed no signal with the antisense *PHAN* probe (Figure 17A), suggesting that the insertion caused a complete loss of *PHAN* transcription. The *PHAN* probe was able to detect signal in wild type apices treated in parallel. Additionally, other antisense probes, e.g. *FLO* (see next chapter) detected signal in *phan*-250G tissue. Therefore the lack of *PHAN* signal on *phan*-250G apices is unlikely reflect the impermeability of the tissue to the probe or inactivity of the probe. Performing the hybridisation with wild type and *phan*-250G apices on the same slide using the *PHAN* probe would not necessarily disprove the lack of signal in *phan*-250G, since sections on the same slide can be exposed to uneven amounts of probe. A consistent lack of *PHAN* signal was seen for *phan*-250G, suggesting that *PHAN* was not being transcribed in this tissue. One way of testing this conclusion is to perform RT PCR (Reverse Transcriptase PCR) with *PHAN* specific primers, which would effectively amplify any *PHAN* mRNA if it was present.

Following the vegetative phase of development, the apex undergoes a transition to become an inflorescence meristem. The inflorescence apex generates bracts in a spiral arrangement. One bract is produced at each node with a floral meristem being produced in the axil of each bract. The floral meristem gives rise to four concentric whorls of sepals, petals, stamens and carpels. Since *phan* mutations also affected bracts and flowers, the expression pattern of *PHAN* in the reproductive phase of development was also investigated. Each longitudinal section through the inflorescence showed several primordia and organs at different stages of development, with earlier stages near the apex and progressively later stages below.

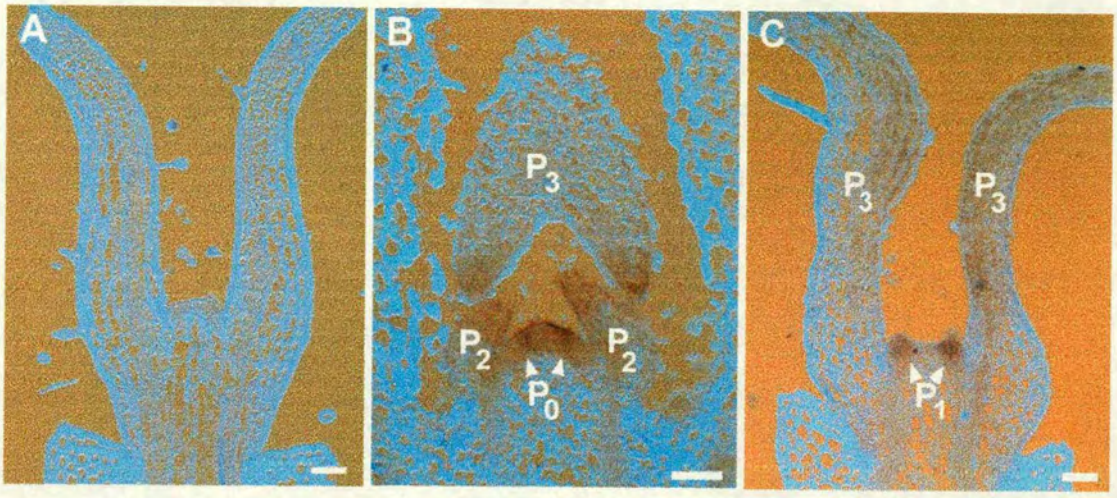


Figure 17. Vegetative apices probed with *PHAN*. Longitudinal sections of wild type shoot tips (B, C) passing through leaf initials at different stages (P_0 etc). No *PHAN* mRNA is detected in apices of *phan*-250G (A). Scale bar is 100 μm.

Due to the spiral phyllotaxy of the inflorescence, only a proportion of the meristems could be observed in each section. In wild type inflorescence apices, *PHAN* showed an early pattern of expression similar to that in vegetative meristems. At the apex, strong expression is seen at bract initial cells (Figure 18B) at least 4 nodes prior to emergence i.e., at I_3 onwards. Upon emergence at P_1, bract primordia still express *PHAN* mRNA strongly. The signal declines rapidly as the bract primordia mature so that after P_{10} signal became undetectable. The signal was uniform throughout all primordia. To put this expression pattern into context, it was compared to the expression pattern of *FLORICAULA* (*FLO*) in adjacent sections (Figure 18C). *FLO*, is a meristem identity gene (Coen *et al.*, 1990) that is expressed in bracts from P_0, so acting as an early marker for bract fate. Unlike *PHAN*, the FLO signal shows some dorsal restriction. This comparison showed that *PHAN* expression was limited to bract cells as marked by *FLO*, and that *PHAN* was expressed at an earlier stage than *FLO*.

PHAN expression was strong in young floral meristems, at the early loaf stage. By the end of the loaf stage, expression was more concentrated at the flanks of the meristem, before sepal primordia become visible (Figure 18D). When the floritypic stage was reached, signal was seen in sepal primordia and strongly marked petal initial cells (Figure 18G). Signal was detected sequentially in each floral organ primordia and declined to become more distal, within floral organs, as they matured (Figure 18L). As in leaves, no dorsal restriction of signal was observed in bracts or floral organs. Inflorescence and floral meristems of *phan*-250G plants showed no *PHAN* signal (Figure 18A), which is consistent with the lack of signal in the vegetative meristems of *phan*-250G.

Because it had been demonstrated that *PHAN* is expressed in all floral organs, the relationship of *PHAN*'s expression pattern to other organ identity genes was investigated. *DEFICIENS* (*DEF*) and *PLENA* (*PLE*) are needed for *b* and *c* functions, respectively. *PHAN*'s expression pattern was also compared to that of

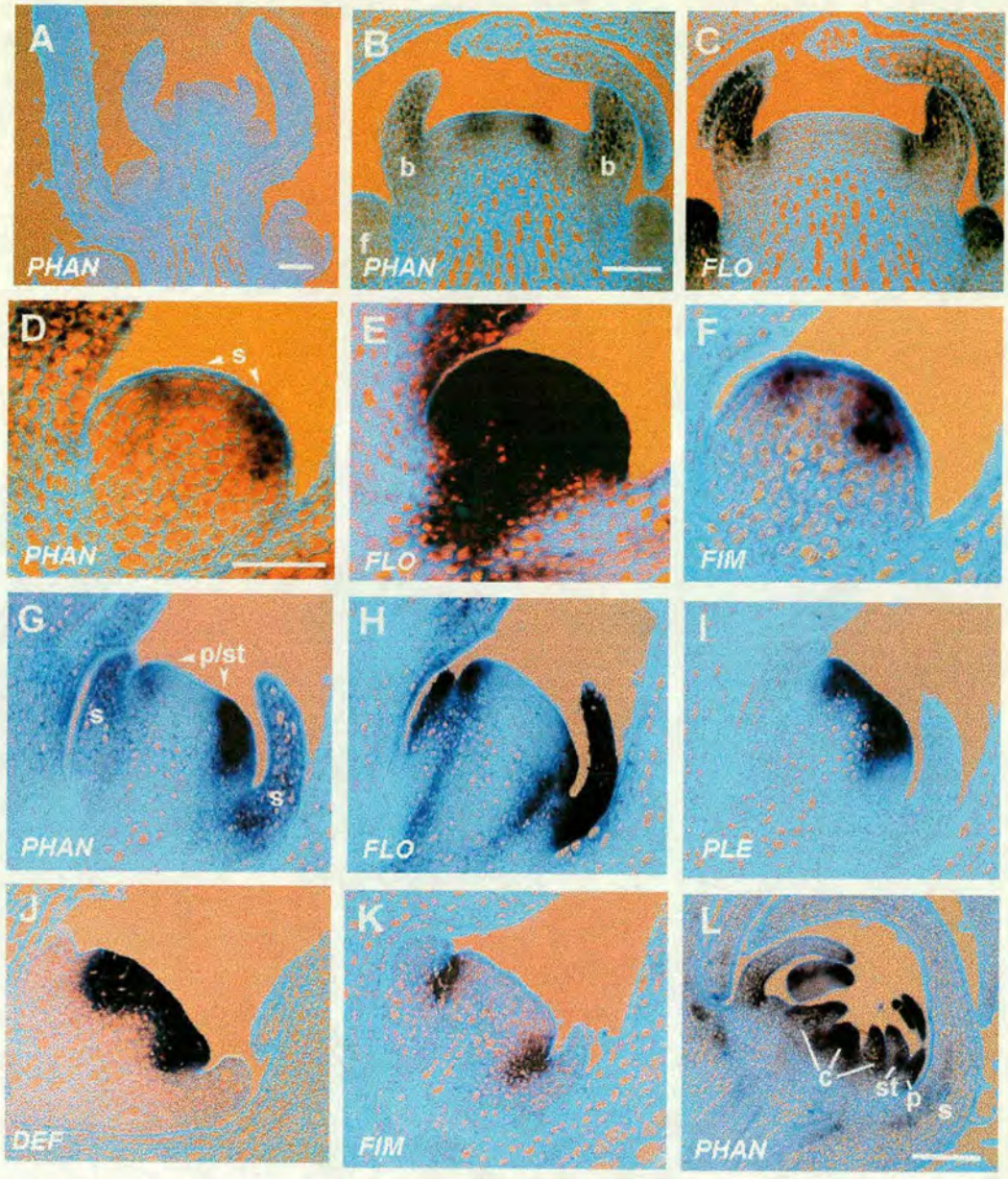


Figure 18. The expression of *PHAN* in inflorescence and floral meristems. Adjacent longitudinal sections of wild type inflorescence apices were probed with *PHAN* (B) or *FLO* (C). In serial sections of young floral meristems, *PHAN* mRNA is detected in sepal initials (D) before *FLO* becomes confined to the same domain (E), while *FIM* expression marks the dorsal region of sepal initials (F). *PHAN* expression (G) is compared to the expression of *FLO* (H), and *PLE* (I) in floral meristems at the floritypic stage in serial sections. Expression of *DEF* (J) and *FIM* (K) can be seen in floral meristems at a similar developmental stage. *PHAN* is expressed in a wild type flower at a later stage (L). There is no *PHAN* signal detected in inflorescence or floral meristems of *phan*-250G plants (A). Scale bars represent 100μm in A, B, G and L and 50μm in D. s, sepal; p, petal; st, stamen; c, carpel; f, floral meristem; b, bract.

FLO and *FIM* which has been shown to mediate between meristem identity and organ identity genes.

FLO marks very young floral meristems at P_0 and is expressed throughout the meristem at the eye stage (Carpenter *et al.*, 1995). During the loaf stage, *PHAN* expression becomes restricted to sepal initials, while *FLO* is still expressed generally throughout the meristem (Figure 18E) and *FIM* is expressed more dorsally in the meristem and then marks the dorsal side of sepal initials (Figure 18F). By the pentagon stage, *FLO* expression is limited to sepal primordia as they just become distinct from the meristem, while *FIM* is expressed more generally in the meristem, till the floritypic stage. At the floritypic stage, *PLE* and *DEF* are expressed in partially overlapping domains of the floral meristem corresponding to initials of whorls two, three and four (Figures 18I, 18J). At this stage, *PHAN* is expressed in sepal primordia and petal initials (Figure 18G), while *FLO* expression is restricted to sepal primordia (Figure 18H). The petal initials are marked by the expression of *DEF* and *FIM* (Figures 18J, 18K). Stamen identity has not yet been defined as being separate from carpel identity, since *DEF* and *PLE* are expressed in the central region of the meristem, so that *PHAN* is not yet expressed in these whorls, but is restricted to sepals and petal initials (Figure 18G). At a later stage, *DEF* expression moves away from the centre of the meristem and the outer boundary of *PLE* expression moves centrally, as stamen identity is defined. This event coincides with *PHAN* expression in stamen initials. Similarly, carpel fate is not determined until a much later stage when *PLE* expression remains in the centre of the floral meristem, after which, *PHAN* and *FLO* are both expressed in carpels. By the floritypic stage onwards, *FIM* expression forms a ring, adjacent to the domain of *FLO* expression in sepals, and which overlaps with *DEF* expression in petal initials (Figure 18K, 18H, 18J). By the petal mound stage and onwards, *FIM* expression is restricted to the boundaries of initial cells as their identity becomes defined and the centre of the meristem while organ identity has not been determined. This is complementary to the expression pattern of *PHAN* which sequentially marks floral organ primordia, while *FIM* marks the boundaries between whorls, as organ identity is assigned.

Floral homeotic genes act sequentially in partially overlapping domains to define organ identity. Comparison of *PHAN* and homeotic gene expression suggests that *PHAN* is expressed in a set of floral organs at or before the stage at which identity becomes defined. There is PHAN expression throughout the floral organ primordia, but as they mature, signal is seen to become weaker, more distal and possibly dorsally localised. The model explaining the role of *PHAN* in determining dorsoventrality (Waites and Hudson, 1995) proposes that *PHAN* acts in the dorsal region of organ primordia. Two aspects of *PHAN*'s expression pattern were not predicted by this model. Firstly, that *PHAN* is not dorsally restricted. This indicates that other spatially restricted factors are required to specify dorsal cell identity. This also raises the possibility that *PHAN* has other, redundant functions in ventral regions. Secondly, *PHAN* is expressed in all lateral organs. However, *PHAN* is only required for dorsoventrality in leaves, bracts and petal lobes, implying that it has redundant functions in organs unaffected by *phan* mutations. *PHAN* mRNA is not detected in either vegetative or reproductive apices of *phan*-250G, therefore this allele can be regarded as an RNA null.

The *phan* Mutant Phenotype is Temperature Sensitive

The *phan* phenotype indicated a role for *PHAN* in determining dorsoventrality in leaves, bracts and petals, while the expression pattern indicated a role in defining lateral organs. A further role in maintaining the meristem was suggested by the temperature sensitivity of the phenotype.

The *phan* mutant phenotype gives, in the extreme, needle like leaves, bracts and petal lobes. This phenotype is sensitive to temperature. All *phan* mutants including the likely null, phan-250G, show this effect of temperature, therefore, it is not an effect on *PHAN*, but an effect on other genes that interact with *PHAN*. In comparison to wild type plants, *phan* mutant plants appear more wild type at warmer temperatures than at colder temperatures (Figure 19). At 17°C the *phan* plants have a very extreme mutant phenotype, in contrast to wild type plants of the same age. At 20°C, the *phan* plants show a less severe phenotype, having broader leaves, bracts and petal lobes. A growing temperature of 25°C gives an appearance very near to wild type.

To determine whether PHAN might have additional functions consistent with its expression pattern, phan mutants were grown at the lower temperature of 15°C. At 15°C, plants failed to germinate, but if, following germination at 20°C, the plants were moved to a growing temperature of 15°C, after they produced one or two pairs of leaves, the meristems of these plants occasionally become quiescent, and no further primordia are produced. The quiescent meristems have a flattened, unorganised appearance (Figure 19), in comparison to wild type apical meristems, which are dome shaped, with primordia regularly emerging from their flanks (Figure 19). This suggested another requirement for *PHAN* in meristem activity and primordial initiation and growth, at the restrictive temperature.

Temperature shift experiments were carried out to determine whether the temperature sensitivity of the *phan* mutant phenotype corresponded to the time of *PHAN* mRNA expression. Two sets of plants were grown, each set consisting of wild type plants and *phan*-607 plants. One set of plants were allowed to germinate at 20°C and were

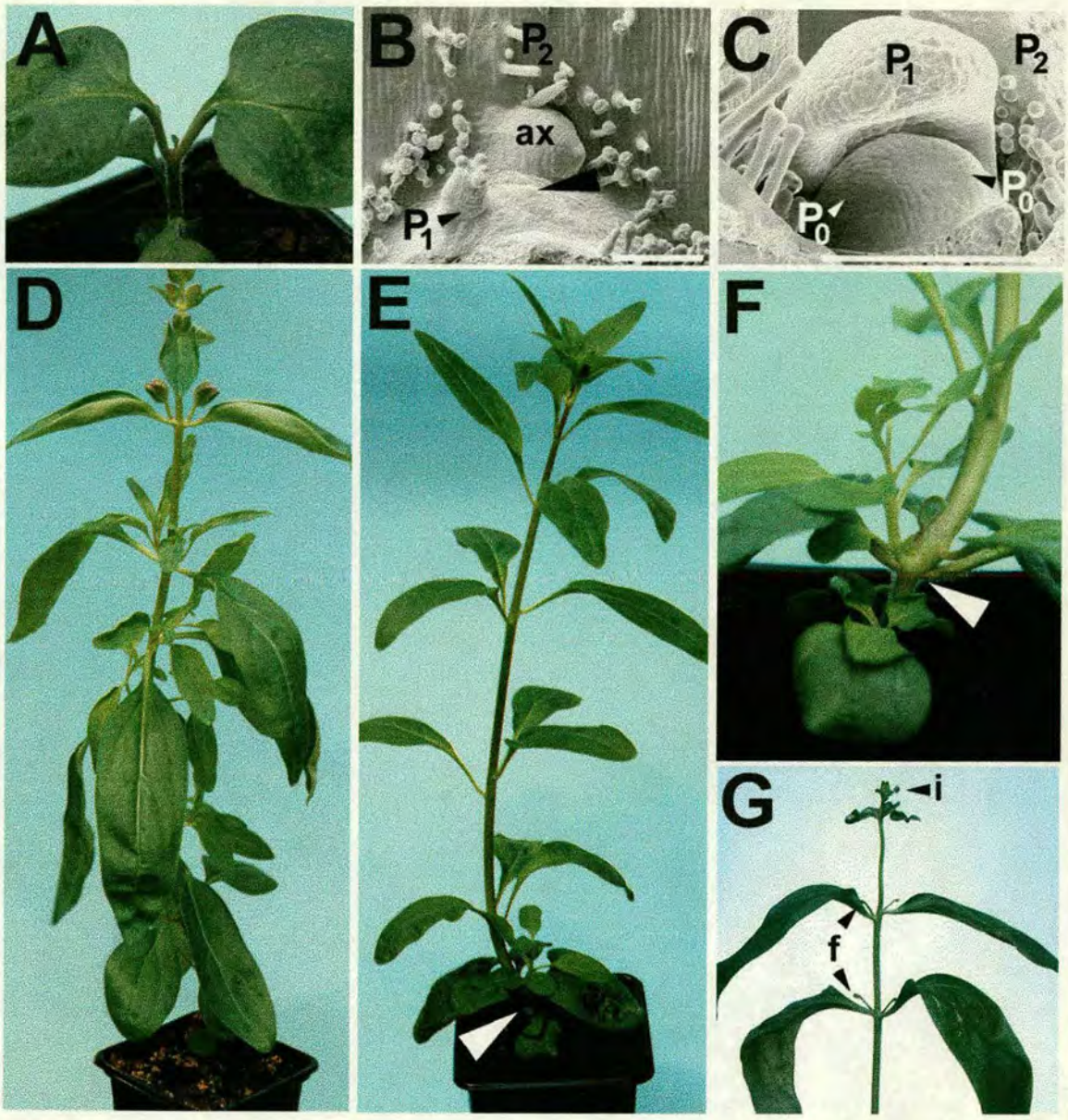


Figure 19. Temperature-sensitive effects of *PHAN* mutations on organ and meristem function. (A) A *phan*-607 plant arrests growth at 15°C. SEM of the apex of such a plant (B) is compared to the apex (C) of a wild type plant of the same age and same growth conditions (D). ax = secondary meristems in a leaf axil, arrows indicate corresponding positions on the wild type apex. Shifting *phan* mutants from 15°C to 25°C allowed the meristem function to resume (E) after an initial disruption in phyllotaxy (F). Here, shifting to the higher temperature (above white arrows) caused the resumption of growth of a secondary meristem, while in other cases, the SAM resumed activity. Shifting plants to the lower temperature later in development (G) inhibited inflorescence (i) and floral meristems (f) as well as growth of floral organ primordia. Scale bar =100μm.

then grown at 15°C. In contrast, the other set of plants were germinated and grown at 25°C. After a period of vegetative growth, the temperatures for both sets of plants were swapped round so that the first set experienced a temperature upshift to 25°C, while the second set had a temperature downshift to 15°C. At the time of the temperature shift, apices from each group, mutant and wild type, were dissected and the developmental stage of the meristems was observed under a dissection microscope. Electron microscopy was also carried out for a clearer picture of the meristem and primordia. The developmental stage was indicated by observing which node was just visible as being separate from the meristem. The morphology of the emerging primordia was also noted, that is, whether they arched over the SAM or whether there was an obvious crease between their dorsal surface and the SAM, and was used to divide the interval between the emergence of successive nodes. Leaf lengths were also measured. It was found that at both temperatures, spiral phyllotaxy occurred at node 11 suggesting that at this point, the SAM switches to an inflorescence identity. Thus, plants that were initiating nodes 7-10, were used for the temperature shifts. At 25°C, the best correlation between leaf length and developmental stage of the meristem initiating nodes 7-10, was found using the length of the leaf at node 4 (Figure 20). The length of the 4th leaf could therefore be used as an indication of the developmental stage of the meristem, without the need for dissection, which would kill the plant. For example, at a 4th leaf length of 5 mm, node 8 was likely to be initiating. Thus, by measuring the length of the leaf at the 4th node, the developmental stage of the meristem of each plant was determined at the time of the temperature shift. At 15°C, the length of the leaves at node three proved to be a better indicator of the initiation of nodes 7-10 (Figures 21 and 22). This was probably due to plants at the lower temperature growing at a slower rate, making the fourth leaf slightly too small to measure accurately. At the restrictive temperature, wild type and *phan*-607 plants grew at a different rate due to the temperature sensitivity of *phan* mutant plants.

The wild type plants in each group showed no phenotypic changes in response to the temperature shifts. *phan* mutant plants experiencing a temperature downshift, went

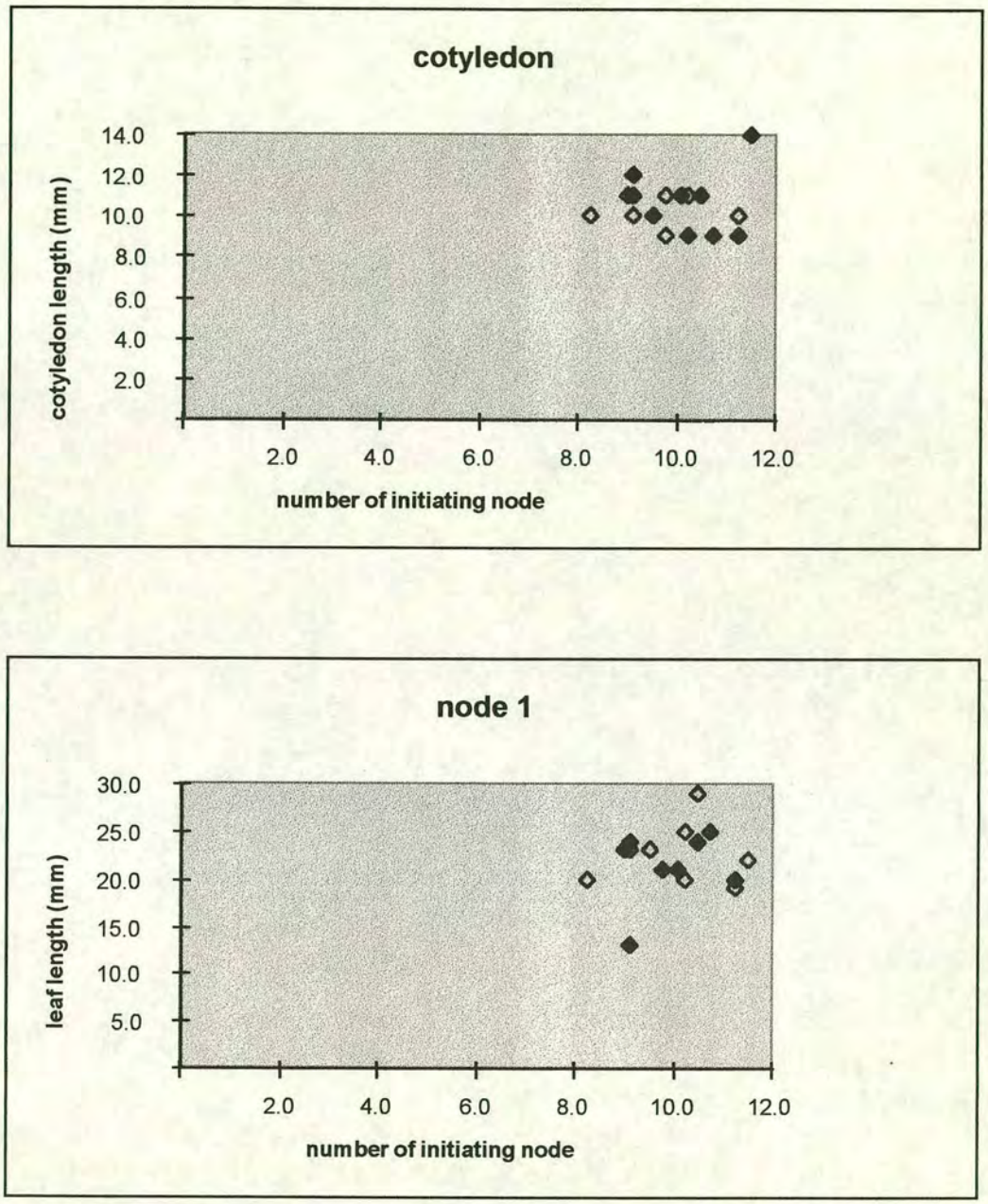
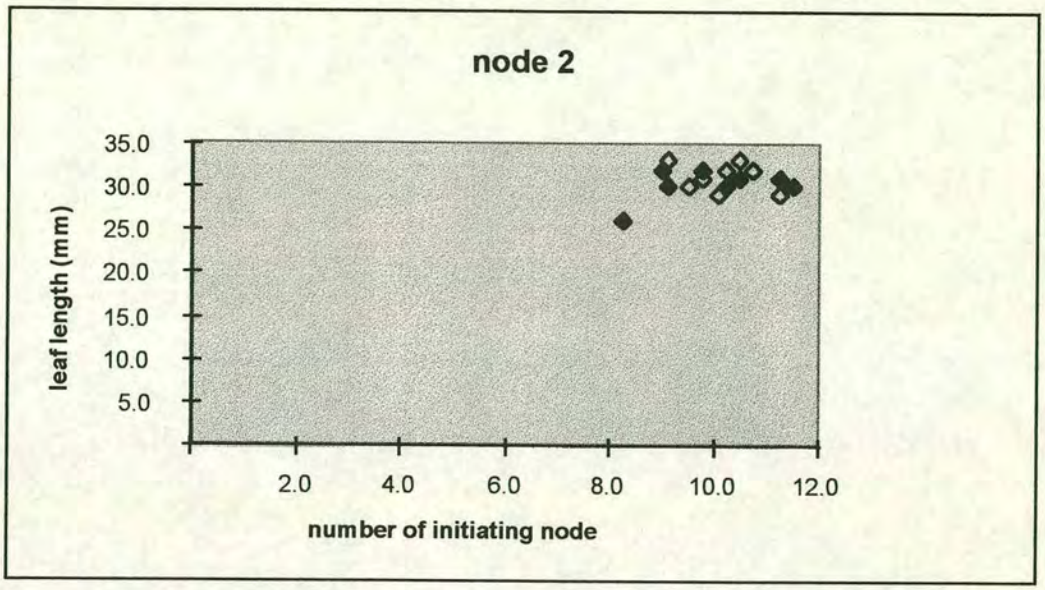


Figure 20a. Developmental stage of wild type (filled) and *phan*-607 (unfilled) plants at the permissive temperature, related to the length of their cotyledons and leaves at node 1.



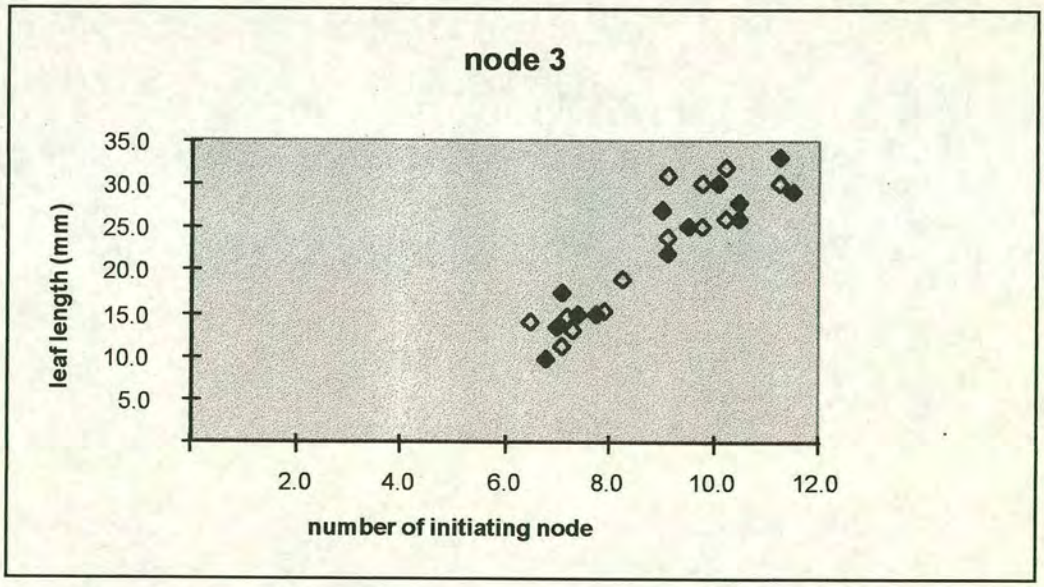


Figure 20b. Developmental stage of wild type (filled) and *phan*-607 (unfilled) plants at the permissive temperature, related to the length of their leaves at nodes 2 and 3.

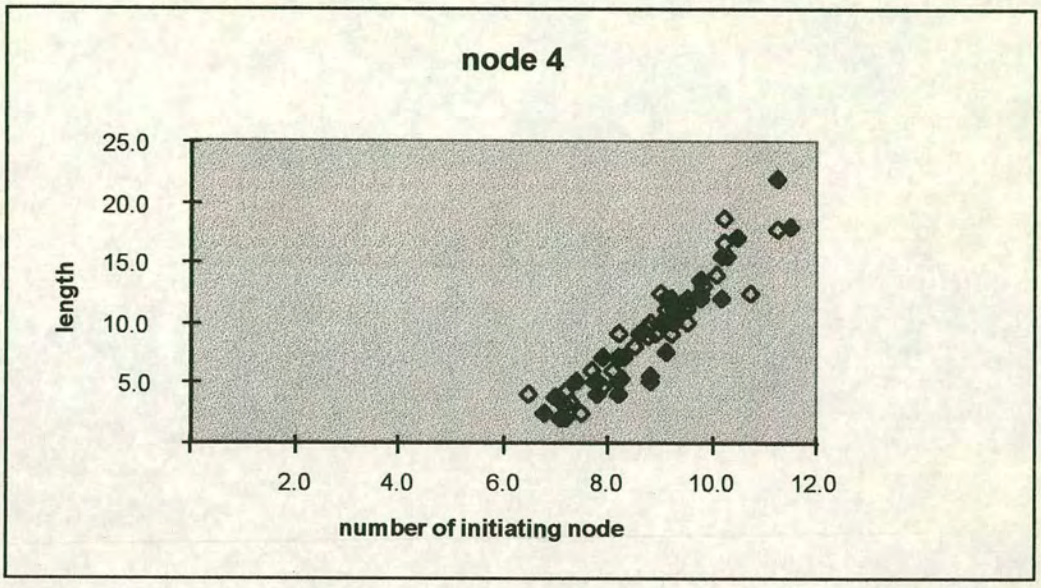
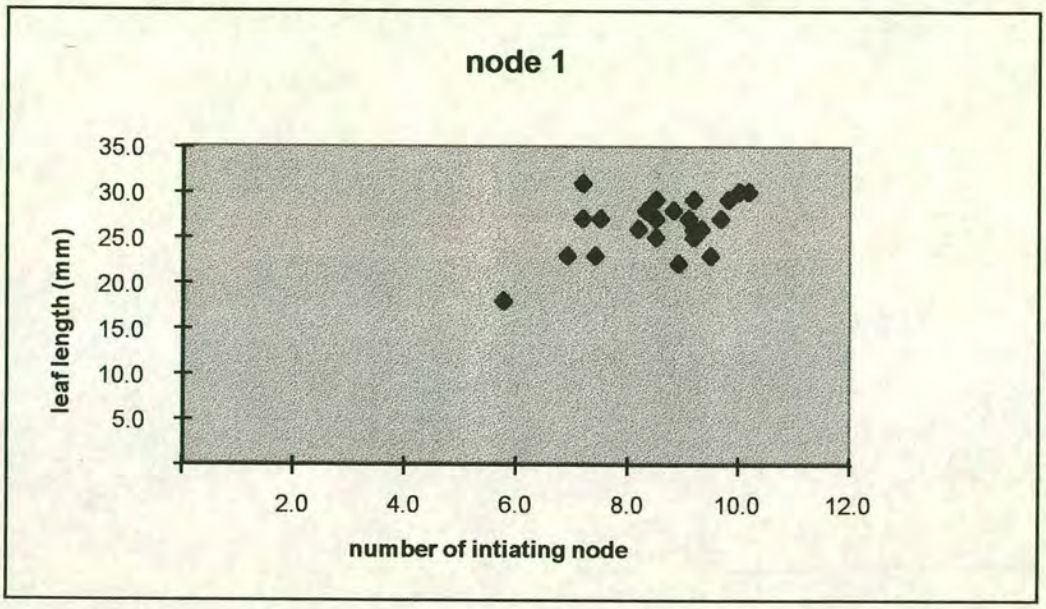


Figure 20c. Developmental stage of wild type (filled) and *phan*-607 (unfilled) plants at the permissive temperature, related to the length of their leaves at nodes 4.



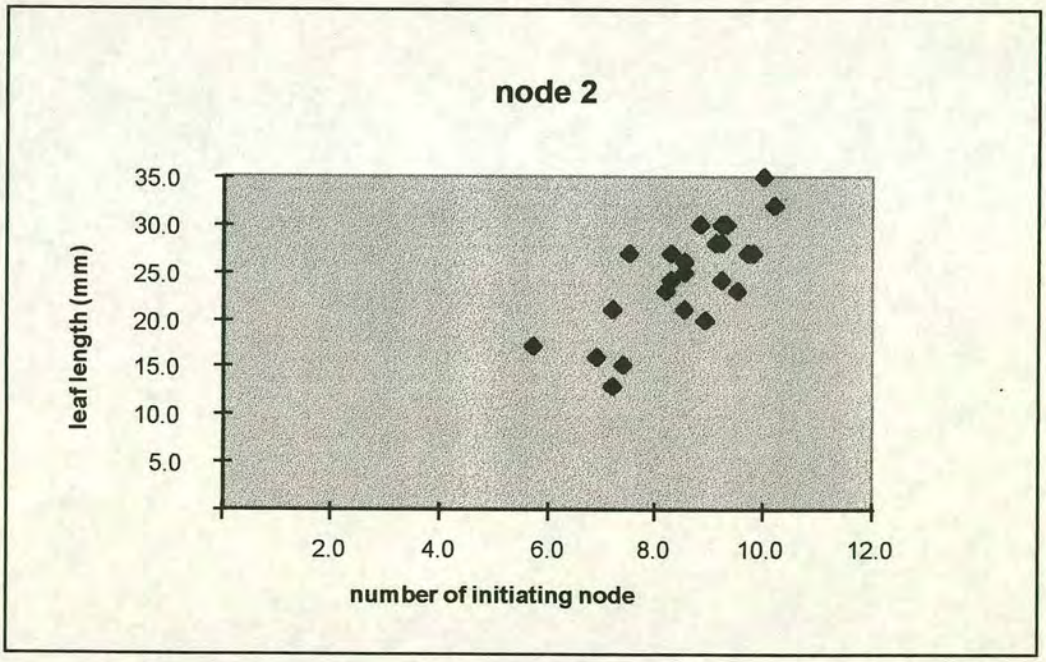
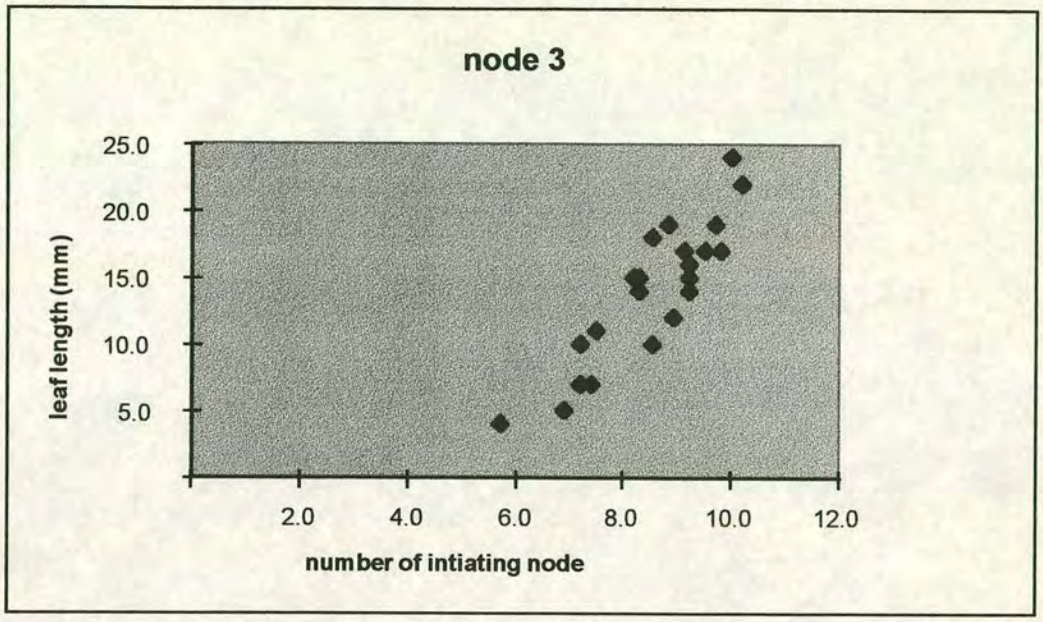


Figure 21a. Developmental stage of wild type plants at the restrictive temperature related to the length of their leaves at nodes 1 and 2.



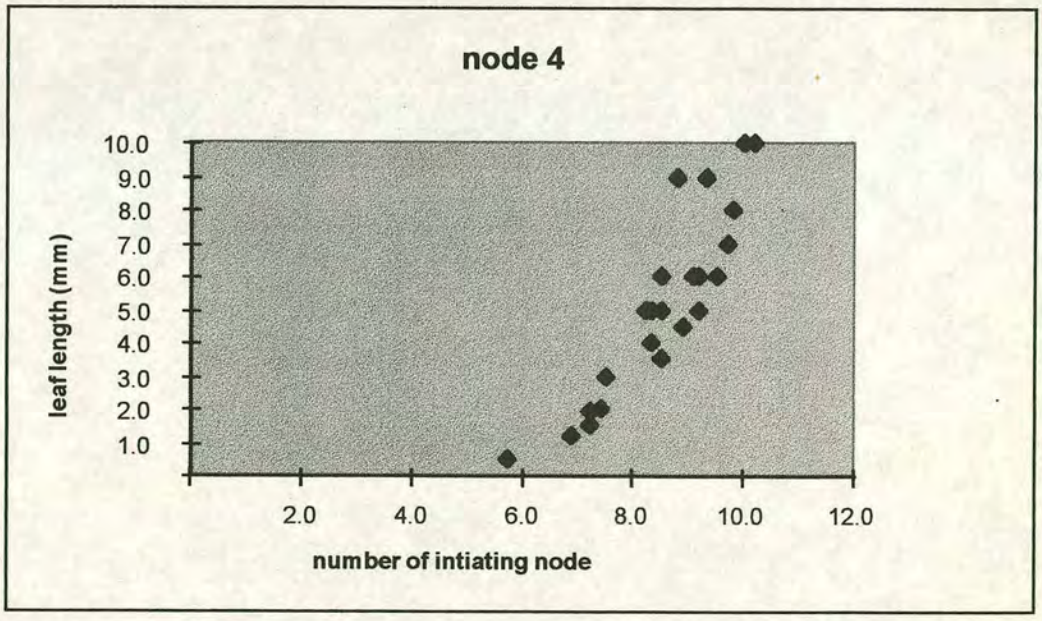
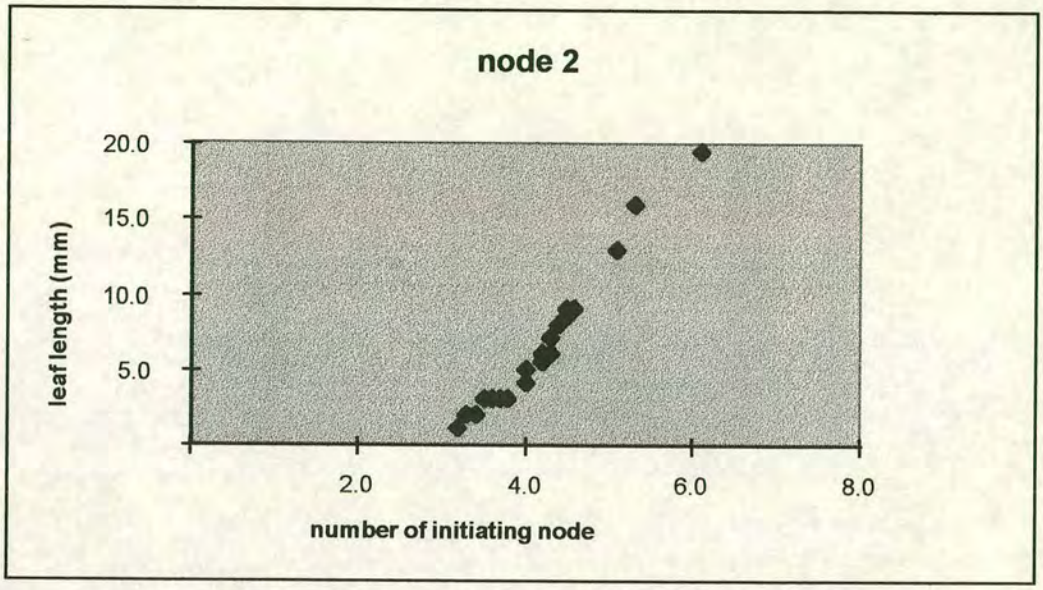


Figure 21b. Developmental stage of wild type plants at the restrictive temperature related to the length of their leaves at nodes 3 and 4.



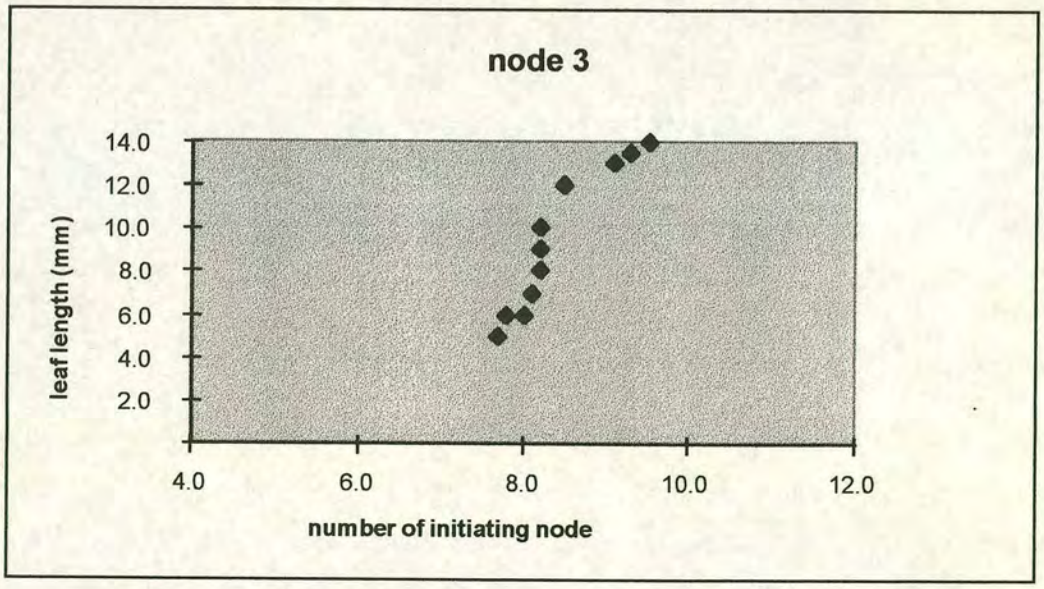


Figure 22. Developmental stage of *phan*-607 plants at the restrictive temperature related to the length of their leaves at nodes 2 and 3.

on to show a more severe mutant phenotype, with both vegetative, inflorescence and floral meristem arrests (Figure 19A, 19B, 19G). Figure 19E shows the effect of a temperature upshift on an arrested meristem. The white arrow indicates the point of the upshift. It can be seen that after an interruption to internode length and phyllotaxy, normal growth resumed and plants went on to flower, suggesting that the meristem was not dead, merely quiescent. It was found that the temperature shift had its effect on the node that was initiating at the time of the shift and on subsequent nodes (Figure 23). For the majority of arrested meristems (61 %), the last leaves to be produced were at either P_0 or P_1 at the time of the shift (Figure 23). For example, in a plant estimated to be initiating node 9 at the time of the down shift, node 9 was the last to be formed normally. Primordia at stages later than P_1 could also go on to produce normal, dorsoventrally flattened leaves. Following the downshift, mature plants initiated primordia for up to four nodes at 15°C, in contrast to seedlings which only produced one or two sets of leaves before meristematic arrest, showing that older plants responded to the increased need for *PHAN* over a longer developmental period.

To investigate the role of *PHAN* on inflorescence and floral meristem development, at the restrictive temperature, *phan* mutant plants were allowed to complete the transition to flowering at 25°C before the temperature shift. A downshift resulted in inflorescence meristem arrest so that further bract primordia and floral meristems failed to initiate. Floral meristems arrested after the production of rudimentary floral organs in a varying number of whorls. The effects of the downshift were visible on floral meristems several nodes after those on bracts, which is consistent with the delay of approximately six nodes between the initiation of bract primordia and the initiation of floral meristems in the axils of those bracts (Carpenter *et al.*, 1995). Thus, *PHAN* is also required for organ initiation and growth, as well as meristem function, in floral and inflorescence meristems, as in vegetative meristems.

phan-607 plants have a reduced level of *PHAN* expression (see next chapter, Figure 27A). The previous chapter showed that *PHAN* was expressed early, prior to lateral organ emergence from the meristem. The temperature shifts showed that *phan*

mutant initials are sensitive to temperature before they become distinct from the meristem. This requirement for *PHAN* is consistent with its expression pattern. At colder temperatures, there is an increased requirement by all apical meristems for *PHAN* expression in organ initials, to maintain primordial initiation and growth, and phyllotaxy, by these meristems. Therefore, *PHAN* seems not only to be required for the formation of lateral organ primordia, but also appears to act non cell-autonomously to play a role in meristem function.

Number of nodes produced after temperature shift	**Number of plants**
0	15
1	46
2	28
3	10
4 +	1

Figure 23. Table to show the effect of a temperature downshift on the node initiating at the time of the shift. The leaf lengths were used as an indication of the number of the node initiating at the time of the shift, such that plants with a leaf length at node 4 of 2-4 cm were assumed to be initiating node 7 and those with leaves of 5-10 cm were assumed to be initiating node 8 (see Figure 20C). These plants were then scored on the number of subsequent nodes produced.

To test the hypothesis that definition of lateral organ identity is required to maintain the meristem and *vice versa*, the expression of genes that are required for meristem function and are expressed in different domains of the meristem and initial cells were compared.

STM is required for the formation of the SAM during *Arabidopsis* embryogenesis. It is also necessary for maintaining functional meristems postembryonically. It is expressed in the meristem and is down regulated in regions corresponding to P_0, so providing the earliest known marker for leaf fate (Long *et al.*, 1996). In order to investigate any relationship between *PHAN* and *STM*, the *STM* homologue from *Antirrhinum* was required. This was obtained by screening an *Antirrhinum* inflorescence cDNA library with an *STM* cDNA clone, kindly provided by Kathryn Barton (University of Wisconsin-Madison, Madison, Wisconsin 53706, USA), at low stringency. The *Antirrhinum* gene, *AmSTM*, had the potential to encode a protein which was more closely related to the *STM* gene product than to the most similar sequence from *Arabidopsis*, *KNAT1*. AmSTM shared 73% identity to the 270 C-terminal amino acids of STM (Figure 25), while KNAT1 shared 54% identity with STM in the same C-terminal region. As with other KNOTTED1-like proteins, AmSTM demonstrated little sequence conservation in the N-terminal region. Therefore, it was likely that *AmSTM* was the orthologue of *STM*. A 450 bp *Kpn* I/*Hinc* II fragment upstream of the well conserved ELK and homoebox domains was subcloned into pBluescript (KS and SK), so that the T7 promoter would give either sense (control) or antisense strands from *in vitro* transcription reactions. The *Kpn* I site was in the polylinker and the *Hinc* II site was at 419 bp from the start of the cDNA sequence (Figure 24). *In situ* hybridisations with the sense strand showed no signal, as expected. When the antisense probe was used, the pattern of mRNA localisation for *AmSTM* in *Antirrhinum* was equivalent to that of *STM* in *Arabidopsis*, where *STM* is expressed in the undifferentiated cells of the meristem, and downregulated in organ initials and organ primordia. To test whether the

```
                                                                    60
TCTCAACAAGGGTTTTCAGACAAAACTTAAGGGCAGAAGCAAAAAAGAAATGGAGGGTAG
                                                      M  E  G  S

                                                                   120
TGGTGGTGGTGGTATGATGGCTTTTGGAGAAAACAATAATAATAATAGTAATAATGGCTT
  G  G  G  G  M  M  A  F  G  E  N  N  N  N  N  S  N  N  G  F

                                                                   180
TTGTCCTAGTAACATGATGATGCCTCAACTGGCTTCTAATTGTGAAGGTGGGTATAGTAC
  C  P  S  N  M  M  M  P  Q  L  A  S  N  C  E  G  G  Y  S  T

                                                                   240
TACTCCATTTGTCCCTCTAGTTCCATTCACAAATCATCATCATCATCATCAAGAGATGAA
  T  P  F  V  P  L  V  P  F  T  N  H  H  H  H  H  Q  E  M  N

                                                                   300
TCGCCAAAGGAGCACAGTTGTTGGAGGTTCCTCTATGGTGTATGAAGGACATCAGAACAA
  R  Q  R  S  T  V  V  G  G  S  S  M  V  Y  E  G  H  Q  N  N

                                                                   360
TAGCAACGCGATCACGAGCACTGGGTACTACTTCATGGACAGTACTAACTGTGATGAAAG
  S  N  A  I  T  S  T  G  Y  Y  F  M  D  S  T  N  C  D  E  S

                                                                   420
CTCTCTCAAGGCAAAGATCATGGCTCATCCTCATTACCATCGACTCTTGGCTGCCTATGT
  S  L  K  A  K  I  M  A  H  P  H  Y  H  R  L  L  A  A  Y  V

                                                                   480
TAACTGTCACAAGATAGGAGCCCCCCCTGAGGTGGTGTCGAGGCTAGAAGAAGCAGCCGC
  N  C  H  K  I  G  A  P  P  E  V  V  S  R  L  E  E  A  A  A

                                                                   540
GGCAATGGCCCGCCACGGCACGATCAGCGTAGGCGAAGATCCGGGGCTGGACCAGTTCAT
  A  M  A  R  H  G  T  I  S  V  G  E  D  P  G  L  D  Q  F  M

                                                                   600
GGAGGCCTATTCTGAAATGCTGTCCAAATATGAGCAAGAACTCTCAAAACCCTTCAAAGA
  E  A  Y  S  E  M  L  S  K  Y  E  Q  E  L  S  K  P  F  K  E

                                                                   660
AGCTATGCTCTTCCTTTCAAGAATTGAGTCCCAGTTTAAAGCCCTCACTGTATCTGCAGC
  A  M  L  F  L  S  R  I  E  S  Q  F  K  A  L  T  V  S  A  A

                                                                   720
TCGTGGCGAAGCAATGTTTAGGAATGGATCATCTGAAGAGGAAATCGACGTGAATAACAG
  R  G  E  A  M  F  R  N  G  S  S  E  E  E  I  D  V  N  N  S

                                                                   780
TTTCATAGACCCTCAAGCAGAAGACATTGAATTGAAAGGTCAGCTCTTGCGAAAATACAG
  F  I  D  P  Q  A  E  D  I  E  L  K  G  Q  L  L  R  K  Y  S

                                                                   840
TGGATATTTGGGAAGCCTCAAACAAGAATTCATGAAGAAAAGAAAGAAGGGAAAGCTACC
  G  Y  L  G  S  L  K  Q  E  F  M  K  K  R  K  K  G  K  L  P

                                                                   900
TAAAGAAGCCAGGCAACAATTACTAGAGTGGTGGAGCCGCCATTACAAATGGCCTTATCC
  K  E  A  R  Q  Q  L  L  E  W  W  S  R  H  Y  K  W  P  Y  P

                                                                   960
TTCGGAATCCCAGAAGCTGGCATTGGCAGAATCAACCGGTCTTGACCAGAAGCAAATAAA
  S  E  S  Q  K  L  A  L  A  E  S  T  G  L  D  Q  K  Q  I  N

                                                                  1020
CAACTGGTTCATTAACCAAAGGAAACGACACTGGAAACCTTCTGAAGACATGCAGTTCGT
  N  W  F  I  N  Q  R  K  R  H  W  K  P  S  E  D  M  Q  F  V

                                                                  1080
AGTGATGGACGCTGCAAATCCGCAATATTACATGGAAAATATTTTGGGTAATCCTTTTCC
  V  M  D  A  A  N  P  Q  Y  Y  M  E  N  I  L  G  N  P  F  P

GATGGATATCTCGCCTGCACTTCTTTGA
M  D  I  S  P  A  L  L  *
```

Figure 24. The cDNA sequence of *AmSTM* isolated from an *Antirrhinum* inflorescence library. The upstream less conserved region was subcloned using a *Kpn* I site in the upstream polylinker while the *Hinc II* site is indicated in magenta. The well conserved ELK and homoebox domains are shown blue and green respectively.

```
        1                                                    50
AmSTM   MEGSGGGGMM AFGENNNNNS NNGFCPSNMM MPQ.LASNCE GGYSTTPFVP
  STM   ME.SGSNSTS CPMAFAGDNS DGPMCPMMMM MPPIMTSHQH HGHDHQHQQQ

        51                                                   100
AmSTM   LVPFTNHHHH HQEMNR.... .......QRS TVVGGSS... ..MVYEGHQN
  STM   EHDGYAYQSH HQQSSSLFLQ SLAPPQGTKN KVASSSPSS CAPAYSLMEI

        101                                                  150
AmSTM   NSNAITSTGY YFMDSTNCDE SSLKAKIMAH PHYHRLLAAY VNCHKIGAPP
  STM   HHNEIVAGGI NPCSSFS.SS ASVKAKIMAH PHYHRLLAAY VMCQKVGAPP

        151                                                  200
AmSTM   EVVSRLEE.. ..AAAAMARH G.TISVGEDP GLDQFMEAYS EMLSKYEQEL
  STM   EVVARLEEAC SSAAAAAASM GPTGCLGEDP GLDQFMEAYC EMLVKYEQEL

        201                                                  250
AmSTM   SKPFKEAMLF LSRIESQFKA LTVS.....A ARGEAMF... RNGSSEEEID
  STM   SKPFKEAMVF LQRVECQFKS LSLSSPSSFS GYGETAIDRN NNGSSEEEVD

        251                                                  300
AmSTM   VNNSFIDPQA EDIELKGQLL RKYSGYLGSL KQEFMKKRKK GKLPKEARQQ
  STM   MMNEFVDPQA EDRELKGQLL RKYSGYLGSL KQEFMKKRKK GKLPKEARQQ

        301                                                  350
AmSTM   LLEWWSRHYK WPYPSESQKL ALAESTGLDQ KQINNWFINQ RKRHWKPSED
  STM   LLDWWSRHYK WPYPSEQQKL ALAESTGLDQ KQINNWFINQ RKRHWKPSED

        351                                  384
AmSTM   MQFVVMDAAN P.QYYMENIL GNPFPMD.IS PALL
  STM   NQFVVMDATH PHHYFMDNVL DNPFPMDHIS STML
```

Figure 25. Alignment of *STM* and *AmSTM* amino acid sequence to show that *AmSTM* is the likely orthologue of *STM*. Identical residues are shown in green.

domains of *PHAN* and *AmSTM* expression were complementary, they were compared by hybridising adjacent sections to *PHAN* or *AmSTM* probes. In vegetative meristems (Figure 26B), *AmSTM* was expressed in undifferentiated meristem cells but was down regulated in the regions corresponding to leaf initials. As with *STM*, no expression of *AmSTM* was detectable in leaf primordia after stage P_1. During reproductive growth, *AmSTM* was expressed in undifferentiated cells of the inflorescence meristem (Figure 26D). Expression was down regulated in domains corresponding to the position of floral meristems and bract primordia. Floral meristems at the loaf stage showed *AmSTM* expression in central regions of the meristem. By the time organ identity genes were being expressed at the floritypic stage, *AmSTM* expression was restricted to the undifferentiated cells in the centre of the meristem (Figure 26F). Figure 26F shows that expression of *AmSTM* is absent in regions where sepal and petal identities have been assigned. The same is true when stamen and carpel fates are assigned. This pattern of expression is complementary to that of *PHAN* which is expressed as cells become differentiated (Figures 26A, 26C, 26E). Adjacent sections were hybridised with either *PHAN* or *AmSTM* probes, to show that their domains of expression do not overlap (Figure 26).

This reciprocal pattern of expression suggests that the two genes may negatively regulate the expression of each other. That is, the expression of *PHAN* in organ initials may lead to the downregulation of *AmSTM* in that domain, which is then sufficient for specifying organ identity, or, conversely, *AmSTM* expression may limit *PHAN* expression to organ initial cells, so specifying organ fate. If *PHAN* is required to negatively regulate *AmSTM* in organ primordia, then there should be ectopic *AmSTM* expression in *phan* mutant initials and primordia. When the probable null allele, *phan*-250G meristems were hybridised with the *AmSTM* probe, there was a normal pattern of *AmSTM* expression, but at a reduced level (Figures 27A, 27E). This suggests that *PHAN* is not required for *AmSTM* expression. However, the consistently reduced level of *AmSTM* signal observed at the apex, may indicate that *PHAN* may be required to maintain *AmSTM* expression in the cells neighbouring the

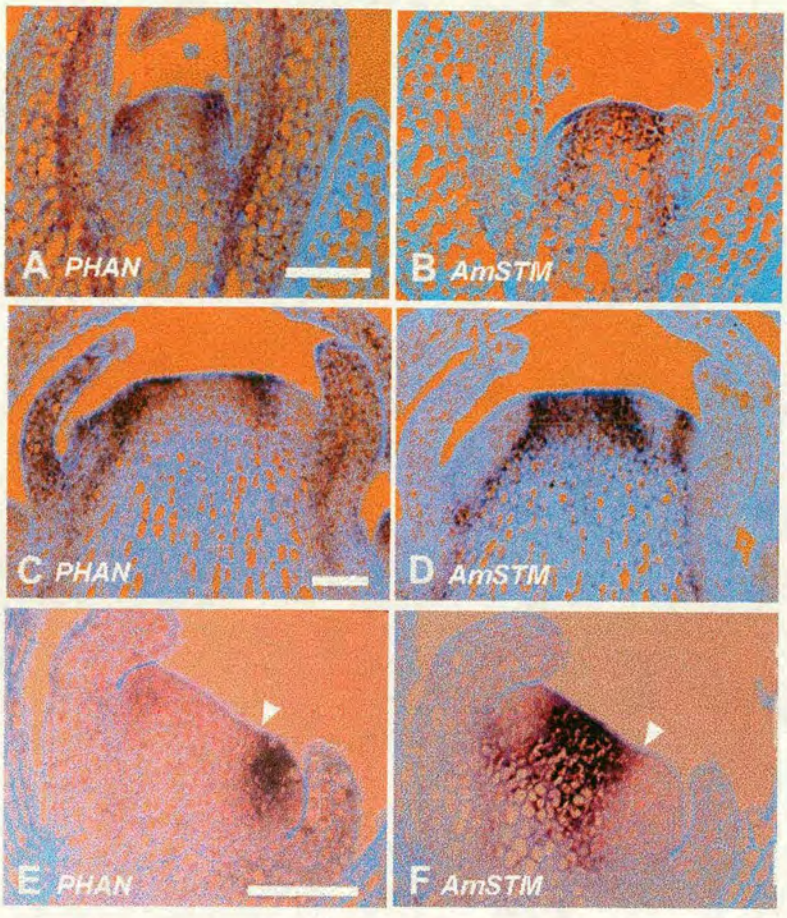


Figure 26. The expression domains of *PHAN* and *AmSTM* are complementary (see arrows). A, C and E show expression of *PHAN* in vegetative , inflorescence and floral meristems, respectively. B, D and F show expression of *STM* in sections adjacent to those in A, C and E. Scale bar = 100 μm.

initials. This is consistent with a non cell-autonomous role for *PHAN* in maintaining meristem activity.

To test whether *AmSTM* might negatively regulate *PHAN*, to prevent expression of *PHAN* in the central undifferentiated regions of the meristem, a mutation of *AmSTM* would be required. To this end, a transposon induced mutation of *AmSTM* has been identified (Andrew Hudson., pers. comm). If *AmSTM* does negatively regulate *PHAN*, then it would be expected that there is ectopic expression of *PHAN* throughout the meristem in *amstm* mutants. Any weak mutants that are identified could be used to test the proposed interaction between *AmSTM* and *PHAN*. If *PHAN* is required to maintain *AmSTM* expression, then *phan* mutations might be expected to exaggerate the phenotype of a weak *amstm* mutation.

Both *STM* and *PHAN* are expressed in meristems. They have been shown to be involved in maintaining functional meristems. How are their patterns of expression affected by meristem arrest? Arrested vegetative meristems were hybridised with *PHAN* and *STM* probes. This revealed no detectable expression of either *PHAN* or *STM* in arrested meristems (Figure 27C). It was found that axillary meristems of these arrested apices, expressed both *PHAN* and *STM* at lower levels (Figures 27D, 27E). *STM* was expressed as in wild type meristems, in the central undifferentiated cells but not in organ primordia. Similarly, *PHAN* was expressed normally in organ primordia and also in the meristem . *FIM* is also expressed as usual, in such meristems. Therefore, *STM* and *PHAN* can be used as markers for functional meristems.

The *NAM* gene from petunia is also required for embryonic SAM formation. *NAM* is also expressed in adult plant tissue, in a domain of the meristem corresponding to the boundary between undifferentiated cells of the meristem and differentiated initial and primordial cells before such separations become visible. *FIM* is also expressed at these boundaries between organ initials and undifferentiated meristem cells, becoming restricted to a ring of expression surrounding the base of organ primordia.

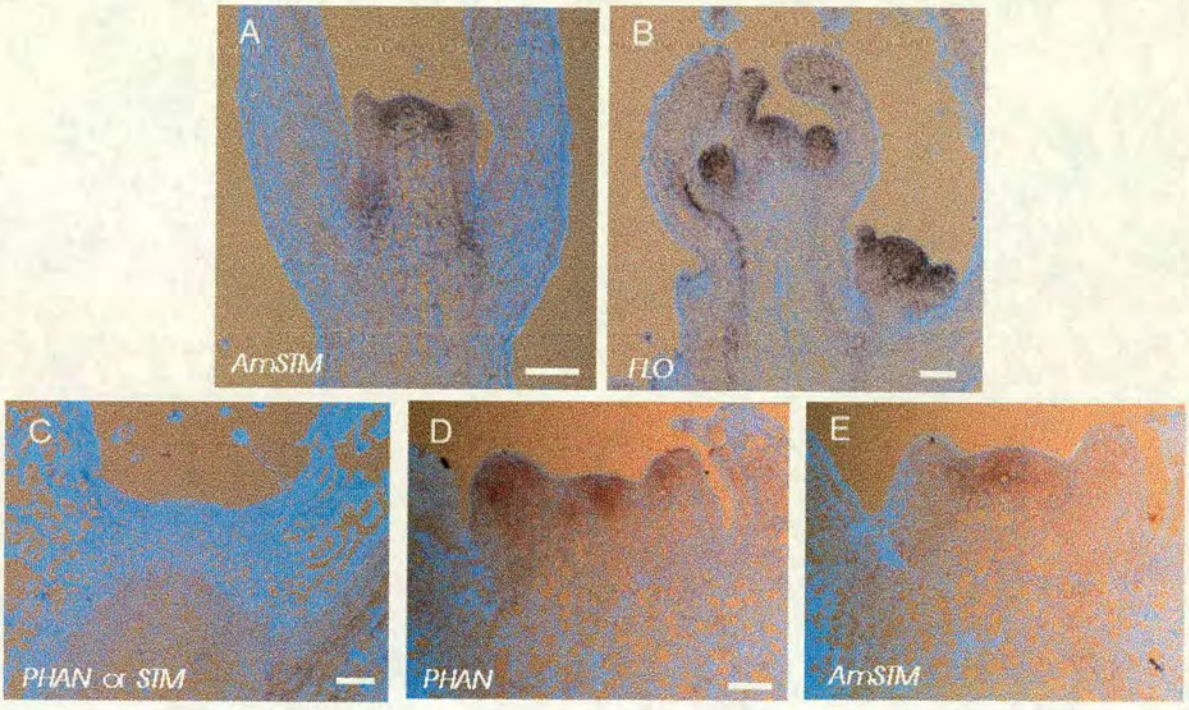


Figure 27. *In situ* hybridisations of longitudinal sections of *phan* mutant apices. *AmSTM* is expressed normally in a *phan*-250G apex (A). Neither *PHAN* or *AmSTM* are expressed in arrested meristems of *phan*-607 (C), however, both *PHAN* and *AmSTM* are expressed in axillary meristems (D, E respectively). *FLO* is expressed ectopically in a *phan*-250G inflorescence apex (B). Scale bar is 100 μm.

It is thought that both *NAM* and *FIM* function to limit cell lineages once organ initial cells have been defined.

NAM and *FIM* are both expressed at the boundaries between organ initials and the rest of the meristem, possibly as a result of their interaction. If *PHAN* is required for organ initial identity, the expression of genes at the boundary may require the activity of *PHAN*. When wild type and *phan*-250G inflorescence meristems were probed with an antisense *FIM* probe, it was found that the pattern of expression was the same in both, but that the level of expression appeared to be reduced in *phan*-250G. This suggested that *PHAN* expression is not required for the expression of *FIM*, but may regulate the levels of *FIM* expression.

To investigate potential interactions between *PHAN* and *NAM*, *NAM*-like sequences were isolated from *Antirrhinum*. Degenerate primers (5'-GARAARGARTGGTAYTTYTT-3' and 5'-TAYTCRTGCATNAYCCARTT-3') were designed using sequences that were well conserved between *NAM* and two *Arabidopsis* cDNA clones, *ATAF1* and *ATAF2*. In *Arabidopsis*, 12 different Nam sequences have been identified (including nine partial sequences of expressed sequence tags) suggesting that they are members of a gene family. Sequences of approximately 240 bp were amplified from *Antirrhinum* genomic DNA using these primers. This DNA fraction was isolated from an agarose gel and ligated into pBluescript that had been digested with *Eco*RV and transformed into *E.coli*. The resultant plasmid preparation was used to re-amplify the *NAM* inserts using M13 -40 and Reverse primers. 11 different sequences were identified (Figure 28). Nam11 was found to be the most similar to *NAM*. It was expected that the expression pattern of the *Antirrhinum* homologue of *NAM* would be similar to that of *NAM* in petunia. In order answer this questions, Nam 11 has been used to make antisense RNA probes, but their use in *in situ* hybridisation experiments proved unsuccessful. Lack of time did not allow this to be pursued.

```
        1                                                  50
nam05   KEWYFFTPRN QKYQNGGRPS RLT..GDGYW KATSGEKKVR YNGEI....I
nam12   KEWYFFTPRN QKYQNGGRPS RLT..GDGYW KATSGEKKVR YNGEI....I
nam09   KEWYFFSTRN RKHHNGSRPN RLTKDDDGFW KATGGNDAVM HNREI....V
nam02   KEWYFFTRRQ KKHANGVRPN R.TTVGNGFW KTTSGDVKIK KGEGE....I
nam08   KEWYFFTRRQ KKHANGVRPN R.TTVGHGFW KTTSGDVKIK KGEGE....I
nam07   KEWYFFTRRQ KKHANGVRPN R.TTVGTGFW KTTSGDVKIK KGEGE....I
nam20   KEWYFFTRRQ KKHANGVRPN R.TTVGNGFW KTTSGDVKIK KGGGE....I
nam10   KEWYFFTQRK EELANGIRPN Q.G..GNGYW KSTGADVKIT IRERE....I
ataf1   KEWYFFSPRD RKYPNGSRPN RSA..GSGYW KATGADKPI. ..G..LPKPV
ataf2   KEWYFFSPRD RKYPNGSRPN RAA..GTGYW KATGADKPI. ..G..KPKTL
nam01   KEWYFFSPRD RKYPNGARPN RAA..TSGYW KATGADKPVL TAG..GTQKV
  nam   KEYWFFSLRD RKYPTGLRTN RAT..EAGYW KATGKDREIY SSK..TSALV
nam11   KEWYFFSVLD RKYGNGAKTN RAT..EKGYW KTTGNDRAVY ..Q..KTQIV
nam04   KEWYFFCKRG RKYRNSVRPN RVT..VSGFW KATGIDRPIY SSGGETRESI
nam03   KEWYFFLHRE QKYRNGSRPN RSA..DRGYL KATDGDKQIG SPE.......

        51                          77
nam05   GFKQVLVFYQ GKRGKAEK.. TNWVMHE
nam12   GFKQVLVFYQ GKRGKAEK.. TNWVMHE
nam09   GSKQVLVYYQ GKHGKAQK.. TNWIMHE
nam02   GRKRTLVYYE GKEKASDK.. TNWVMHE
nam08   GRKRTLVYYE GKEKASDK.. TNWIMHE
nam07   GRKRTLVYYE GKGKASDK.. TNWIMHE
nam20   GRKRTLVYYE GKGRASDK.. TNWIMHE
nam10   GRKKVLVYYE GKEKDRSQVM TNWIMHE
ataf1   GIKKALVFYA GKAPKGEK.. TNWIMHE
ataf2   GIKKALVFYA GKAPKGIK.. TNWIMHE
nam01   GVKKALVFYG GKPPKGIK.. TNWIMHE
  nam   GMKKTLVFYR GRARKGEK.. SNWVMHE
nam11   GMKKTLVYHI GRAPKGQR.. TNWVMHE
nam04   GLKKSLVYYR GSAGKGTK.. TNWVMHE
nam03   SIKMALVFYS DKAPRCEK.. TNWIMHE
```

Figure 28. The predicted amino acid sequence of 11 DNA sequences isolated from *Antirrhinum* (nam 01-05, 07-12) aligned to *NAM* and two *Arabidopsis* sequences (*ATAF1* and *ATAF2*). Residues identical in the majority are shown in green.

FLO is also expressed in the SAM, marking bract initials at P_0 and very young floral meristems at P_1. In floral meristems, *FLO* is expressed in sepal, petal and carpel initials before primordial emergence. *PHAN* is also expressed in lateral organ initials at a similar developmental time. Therefore, it is possible that *FLO* expression in organ initials is necessary for the expression of *PHAN*, or *vice versa*. To test for an interaction between the two genes, *phan*-250G inflorescence meristems were probed with a *FLO* probe. *FLO* was ectopically expressed throughout the SAM and floral meristems (Figure 27B), instead of being confined to bract initials, young floral meristems, and sepal, petal and carpel initials. Thus it seems that *PHAN* expression is required to limit *FLO* expression to organ initials. That is, *PHAN* negatively regulates *FLO* expression in the rest of the meristem. Because this repression of *FLO* in wild type plants by *PHAN* appears to be non cell-autonomous, *FLO* is presumably an indirect target of *PHAN*.

Discussion

The *PHAN* gene Encodes a MYB-Related Transcription Factor

Plant MYB transcription factors

Structure of *phan* alleles

Transposition and gene evolution

The Expression Pattern of *PHAN*

PHAN is expressed early in all lateral organ development

PHAN is expressed ubiquitously in all organ primordia

Genetic redundancy in plants

PHAN Functions to Maintain the Meristem

The *phan* phenotype is temperature sensitive

Other genes involved in meristem function

Organogenesis from the SAM

Leaf Development in *Antirrhinum* and Maize is Analogous

Plant MYB transcription factors

MYB proteins are characterised by the MYB DNA binding domain which consists of two or three tandem repeat sequences at the N-terminus and a C-terminal region which is not conserved. In common with other plant MYBs, PHAN lacks the first repeat (R1), which has been implicated in the negative regulation of DNA binding (Luscher *et al.*, 1990; Tanikawa *et al.*, 1993). The second and third repeat have been shown to be involved in DNA binding (Luscher and Eiseman, 1990). Each repeat consists of three alpha helices arranged in a helix-turn-helix-like structure with conserved tryptophans forming a hydrophobic core (Ogata *et al.*, 1992). Like other plant MYBs, PHAN has these regularly spaced tryptophan residues. However, PHAN differs from other plant MYBs by showing little conservation in the C-terminal region of R3 and also has a slightly longer R2 which has only one residue upstream of it, instead of a leader sequence.

Although redox regulation has not been demonstrated in plants *in vivo*, the Cys43, which has been proposed to play a role in redox regulation of *c*-MYB (Myrset *et al.*, 1993), is also present at a similar position in PHAN. Serine and threonine residues at the C-terminus could also function in a phosphorylation mediated form of regulation (Moyano *et al.*, 1996).

There is at least one other PHAN-like protein in *Antirrhinum* (R. Waites, pers. comm) which shares the C-terminal characteristics of *PHAN*. In Maize, the *ROUGH SHEATH 2* (*RS2*) gene shows high homology to *PHAN* and has been shown to play a role in leaf development (J. Langdale per. comm). Other *PHAN*-like genes have been identified from *Arabidopsis*, tomato and tobacco (Figure 8). The only other similarity is to the product of a gene induced in tomato upon infection by root-knot nematode (Bird and Wilson, 1994). The infection induces the formation of a novel cell type and the ectopic expression of many genes (Opperman and Cronkling, 1994), indicating that this *PHAN*-like gene may also be involved in regulating gene expression to

specify cell identity. These results suggest that *PHAN* is a member of a small sub-family of *MYB* genes.

Structure of the *phan* alleles

The *phan* mutations that had been identified all conditioned the same vegetative phenotype while they all gave a characteristic floral phenotype (Waites and Hudson, 1995). This suggested that they were hypomorphs. Characterisation of the structure of each allele confirmed that all but one was due to an insertion of a transposon within the *Bgl* II fragment.

The most severe allele, *phan*-250G, was found to have an insertion immediately upstream of the start of translation which potentially leads to the termination of transcription upstream of the coding region. This correlated with the extreme floral phenotype of *phan*-250G in which the petal lobes are reduced to needles. No *phan* mRNA was detected in *phan*-250G apices, suggesting that this was the case and that this allele was an RNA null mutation.

phan-249G gave a less severe floral phenotype than *phan*-250G. *phan*-249G was found to carry an insertion of Tam 2 within the coding region. This potentially resulted in the formation of a novel protein which was at least partially functional. It is possible that the insertion was within the transactivation or negative regulation domain. This region has been implicated in regulating the activity of MYB proteins (Dubendorff *et al.*, 1993), so that an insertion within this domain could have resulted in a protein that was mis-regulated. However an insertion within the negative regulation domain would be expected to give a gain-of-function mutation. Alternatively, a substitution of a less efficient sequence in the transactivation domain, by Tam2, could have resulted in a protein with a reduced transactivation ability.

phan-709 conditioned an intermediate floral phenotype and was deduced to contain an insertion within the coding region, similarly to *phan*-249G, or within the 3′

untranslated region. It is likely that this resulted in the formation of a truncated product which is partially functional or mis-regulated.

phan-607 was deduced to be caused by an insertion within the intron, upstream of the start of transcription, which seems to have disrupted splicing to potentially result in a reduced level of protein being formed. This is consistent with the intermediate floral phenotype of *phan*-607 and the reduced level of transcript detected in *in situ* hybridisation experiments.

Insertion of the *Mu1* transposable element into the first intron of the *Adh1* gene of maize results in the accumulation of about 40% of the wild type message (Strommer *et al.*, 1982; Bennetzen *et al.*, 1984). This is due to the termination of most of the transcripts within the *Mu1* element (Vayda and Freeling, 1986; Luehesen and Walbot, 1990; Ortiz and Strommer, 1990). Previously, *phan*-552 was shown to have an insertion of Tam 4 also within the intron. However, the phenotype of *phan* -552 is less severe than *phan*-607, suggesting that either the site of insertion within the intron, or the identity of the transposon has an effect, so that in the case of *phan*-607, the product was even less abundant. This has been shown in maize where the *Spm* element and its gene product suppress or partially activate genes into which they have inserted, depending on the position of the insertion (Grant *et al.*, 1990).

It is possible that there is more than one product of the *PHAN* locus. Two transcription start sites were detected with the upstream position being taken as the major start site, and alternate splice acceptor sites were identified by 5' RACE. Insertions within the intron may differentially affect the processing of the transcription products. The *R-s* subcomplex of maize has shown variable splicing of the leader intron. The *R-r;std* allele of the *r* locus causes pigmentation of the aleurone of the seed as well as in other parts of the plant like the anthers and leaf tips (Stadler, 1942). Cloning of the *R-r;std* allele revealed that it was made up of two components, the *R-p* and *R-s* subcomplexes (Walker *et al.*, 1995). *R-p* activates pigmentation in plant tissues while *R-s* regulates pigmentation in the seed. Within *R-s* there are two leader sequences (termed s) which contain features typical of transposons. It was found that

the intron upstream of the initiation codon was spliced out of transcripts that accumulated in the embryo, while in the aleurone, unspliced message containing s (shown to be a aleurone specific enhancer) as well as spliced message with two different acceptor sites, was detected (May and Dellaporta, 1998). So differential splicing of the intron may account for the tissue specific expression of pigment, although it could also be explained by transposon excision. Analysis of leader sequences of numerous proto-oncogenes has revealed the presence of an additional ATG codon (Arrick *et al.*, 1991; Kozak, 1987), such that the translational suppression of regulatory factors by upstream ORFs seems to be a common process. *PHAN* contains upstream ORFs which may be involved in such a process of regulation by translation. It is interesting to note that the *PHAN* homologues from *Arabidopsis* and maize also have a 5′intron.

In contrast to the other alleles, *phan*-250G and *phan*-249G carry stable transposon insertions so that there are no reversion events. Both have been shown to be germinally stable and there have been no reports of transposons which are germinally stable, but somatically unstable. Most genomic DNA extractions were made from leaves which are more wild type in appearance, i.e. narrow or mosaic leaves, rather than from needle-like leaves. However, no wild type *PHAN* DNA has been detected by Southern analysis of extracts from *phan/phan* tissue. In addition, the effect of temperature on all alleles is reversible at all stages of development. This would not be consistent with somatic reversion events during development, which would result in no temperature sensitivity by parts of the plant where a somatic reversion event had occurred, and the effects of reversion would not be reversible. Therefore, the two alleles can be regarded as stable, both germinally and somatically.

Transposon excision has often resulted in the production of stable alleles due to the creation of footprints so that there is imprecise restoration of the excision site (e.g. Almeida *et al.*, 1989), as can aberrant transposition events which can result in deletions, inversions and insertions (e.g. Bollmann *et al.*, 1991). CACTA transposons like Tam 2, need the activity of Tam 1 to encode a transposase to catalyse their transposition. It is possible that these plants lack an active copy of Tam

1 in their genome or carry some other stabilising/repressor factor. An alternative explanation is that *phan*-250G carries a copy of Tam 3 (which normally provides its own transposase) but has become defective. The *STABILISER* (*ST*) allele, when homozygous, has been shown to dramatically reduce the excision frequency of Tam 3 (Carpenter *et al.*, 1987; Fincham *et al.*, 1967). Stable mutation have also been the result of the insertion of large retrovirus-like elements (Grandbastien *et al.*, 1989) For example RAM 1 (Retrotransposon *A. majus* 1) does not excise upon transposition, but replicates itself via reverse transcription, so that the original copy remains inserted within the donor gene (S. Doyle, 1997). Methylation is also a possible reason for the inactivation of transposons, for example, the inactivation of the *Spm* element in the *a-m2* allele of maize is associated with its hyper methylation (Banks *et al.*, 1988).

It is interesting to note that none of the alleles showed insertions within the MYB DNA binding domain. This is also the case for the *c1* genes from maize which was also cloned by transposon tagging (Paz-Ares *et al.*, 1986; Cone *et al.*, 1986). 7 insertions were identified within the *c1* locus, none of which were within the two MYB repeats (Paz-Ares *et al.*, 1987) Perhaps this reflects transposon preferences for insertion in areas other than the MYB domain. It may however reflect the relatively small proportion of *PHAN* which makes up the MYB domain, so that it is less likely to have accumulated insertions than other larger parts of the locus. The size of the upstream intron also needs to be determined in order for the relative size of the MYB domain to evaluated. It is also possible that all the alleles including the RNA null, *phan*-250G, have a basal level of PHAN activity which is required for the survival of the seedling. Immunolocalisation studies need to be performed to determine if the lack of transcript detected in *phan*-250G also results in a lack of protein. An insertion within the MYB domain might completely knock out the activity of any protein which may be present, and be lethal. The insertion of Tam 2 and subsequent protein substitution at the C-terminal region, in *phan*-249G seems to allow a partially functional or mis-regulated product to be made. This may indicate that this less conserved region is less vital to the function of the protein than is the MYB domain, and so is more tolerant of mutations.

Similarly, no insertions have been found to lie immediately downstream of the MYB repeats. In the *c* locus, an insertion in this position is associated with the *C1-I* allele which is a dominant negative mutation (Coe, 1962; Paz-Ares *et al.*, 1990). This is assumed to be due to the expression of a truncated protein which lacks the downstream activation domain. This truncated protein would be able to compete for DNA binding sites on *C1* controlled genes, but unable to activate them, so blocking the activation of anthocyanin biosynthesis genes Paz-Ares *et al.*, 1990). Other dominant negative mutations have been induced by the expression of only the MYB domain in transgenic plants (Malcolm Campbell, Forestry Dept , University of Oxford, pers. comm.). Insertions within the coding region, as in, *phan*-249G and *phan*-709, are therefore assumed to be outside of the activation domain, since they do not condition dominant negative phenotypes. This raises the question of why no dominant negative mutations of *PHAN* have been identified. Perhaps, as discussed above, binding of the MYB domain only would block the action of interacting proteins to result in lethality or an extreme phenotype.

Transposition and gene evolution

Contrary to the view that transposons are selfish DNA which replicate within an organism without the need for a function, it seems that transposons may act as mobile enhancer/suppresser elements. In the case of the maize *R* complex, transposition has resulted in the re-arrangement of sequence and the gain of new expression patterns (May and Dellaporta, 1998). CACTA like sequences have been found in the 5' region of the *R-s* gene and in alleles of the maize *pl* gene (Cone *et al.*, 1993). Indeed, the characterisation of *phan* mutants has demonstrated that different transposon insertions within the same locus have resulted in subtle variations in flower shape. Thus, transposition may play an important role in introducing novel regulatory elements, resulting in the novel expression patterns of duplicated copies of genes.

The Expression Pattern of *PHAN*

It is clear that *PHAN* plays an important role in the regulation of *Antirrhinum* development. To reveal how it carries out these functions at the molecular level, the expression pattern of *PHAN* was characterized using RNA *in situ* hybridization. This revealed that *PHAN* is expressed early in the development of all lateral organs and that expression is not dorsally restricted.

PHAN is expressed early in lateral organ development

In situ hybridization experiments revealed that *PHAN* is expressed in all lateral organ primordia at a very early developmental stage (P0), before they become morphologically distinct from the meristem at stage P1. Surgical experiments have suggested that the identity of leaves is irreversibly specified during the plastochron before emergence (P0). Snow and Snow (1933) showed that vertical incisions which bisected prospective leaf sites in *Lupinus*, caused the displacement of leaf initiation only if the incision was made at least half a plastochron before primordial emergence. After this time, it seems that leaf initiation sites are fixed. Comparing the timing of *PHAN* expression to other genes which act as markers of floral organ fate *(FLO, FIM, DEF* and *PLE)* also suggests that *PHAN* has an early role in bract and floral organ development. Therefore, *PHAN* expression coincides with the determination of lateral organ identity. The *phan* mutant phenotype indicated that *PHAN* is involved in specifying dorsal identity (Waites and Hudson, 1995). In *Antirrhinum*, dorsoventral asymmetry first becomes apparent in lateral organs after they have emerged from the meristem and start to proliferate along the proximodistal axis. However, surgical experiments have suggested that in a number of species, dorsoventrality is specified within the SAM (Snow and Snow, 1959; Hanawa. 1961). For example, Sussex (1955) found that isolation of the I_1 from the rest of the meristem of potato resulted in the formation of a determinate organ which was radially symmetrical. This would mean that the specification of dorsoventral asymmetry, coincides with the early expression pattern of *PHAN* within the SAM. The temperature shift experiments confirm that *PHAN* functions in determining dorsoventrality before primordial

emergence, since initials that have not emerged at the time of the shift are sensitive to temperature.

Therefore, both the determination of lateral organ identity, and the specification of dorsoventrality, occur prior to any visible morphological differences, which corresponds to the time and position of *PHAN* expression. This indicates a role for *PHAN* in the above two processes. Perhaps these two functions are inseparable. Indeed, excised leaf primordia in culture undergo a period of radially symmetrical growth after which some can become dorsoventral (Steeves, 1962), which suggests that dorsoventrality is a inherent consequence of leaf determination. But, taken together with the results of the surgical experiments described above, it can also be hypothesized that the determination of a leaf could consist of various steps. These steps may be induced sequentially and possibly independently (Steeves and Sussex, 1991).

PHAN is expressed ubiquitously in all organ primordia

Waites and Hudson (1995) proposed a model for the action of *PHAN* in specifying dorsal cell identity, as part of a dorsalising function (DF). DF was proposed to be active in the dorsal part of the leaf, bract and petal primordia. Therefore, it was expected that *PHAN* might be expressed only in the dorsal regions of these primordia. However, it was found that *PHAN* mRNA was detected ubiquitously in all lateral organ primordia. The mutant *phan* alleles, including the RNA null, *phan*-250G, show a loss of dorsal cell identity. This would indicate that although *PHAN* is expressed in all cells of the primordium, it is only required in the dorsal cells of the primordium. Similarly, *PHAN* is expressed in all lateral organs, not just in leaves, bracts and petals, where a loss of dorsal cell phenotype is seen. This means that again, *PHAN* expression may be redundant in sepals, stamens and carpels. Therefore, there are three aspects of *PHAN* redundancy. Firstly, in ventral cells, and secondly, in floral organs except bracts and petal, both with respect to the specification of dorsal identity. Thirdly *PHAN* is redundantly involved in meristem function. The term 'redundancy' is used here to mean that while a gene *X* may be required to function in

one process, it may also be active in another process which is primarily controlled by other genes, such that gene *X* is not required. The gene is therefore redundant in that second process. This genetic redundancy leads one to assume the involvement of other factors.

Genetic redundancy in plants

One of the best characterized examples of genetic redundancy in plant development is the *APETALA2* (*AP2*) gene of *Arabidopsis* which was shown to control floral development by playing a role in (i) establishment of the floral meristem (e.g. Bowman *et al.*, 1993), (ii) specification of floral organ identity (Komaki *et al.*, 1988; Bowman *et al.*, 1989; Kunst *et al.*, 1989), and (iii) the spatial regulation of floral homeotic gene expression (Bowman *et al.*, 1991a; Drews *et al.*, 1991a). However, when its expression pattern was characterized, it was shown that *AP2* mRNA was not restricted to or within developing flowers (Jofuku *et al.*, 1994). *AP2* mRNA was detected in the vegetative shoot meristem, leaves and stem. Elevated levels were detected as the apex became an inflorescence meristem, in young floral meristems and in all developing floral organs. The ABC model for floral organ identity (Coen and Meyerowitz 1991) predicts that *AP2* and *AGAMOUS* (*AG*) are mutually antagonistic in order to restrict *AP2* function to sepals and petals. Jofuku *et al* (1994) showed that *AP2* activity is not determined by *AG* and suggested that *AP2* functions to negatively regulate *AG* transcription in sepals and petals through a partnership with *AP1* and *LEAFY* (*LFY*). Therefore, *AP2* was found to genetically redundant in floral whorls other than whorls one and two. *AP2* is expressed in vegetative tissues, although *ap2* mutant plants do not show any vegetative phenotype. Since *AP2* belongs to a divergent multigene family, it is likely that *AP2*-like genes may be active in vegetative parts, such that *AP2* is redundant during vegetative development (Jofuku *et al.*, 1994). An alternative explanation is that the effects on vegetative growth in *ap2* mutants is sensitive to soil conditions, such that any vegetative phenotype has not been observed in standard growth conditions. (Jofuku *et al.*, 1994).

Another example of genetic redundancy is the action of the *DORSAL* (*DL*) gene of *Drosophila melanogaster*, where differentiation along the dorsoventral axis of the embryo is defined by a signal that specifies ventral fate (Morisato and Anderson, 1995). *DL* is required for ventral identity in the embryo, but is transcribed in the nurse cells (during oogenesis) and translated in the embryo, dorsally and ventrally. Polarization events early in the development of the egg results in the secretion of SPATZLE which is cleaved ventrally by ventral follicle cell products. Following proteolysis, SPATZLE acts as a ligand for the *TOLL*-encoded receptor. After fertilization, SPATZLE triggers the TOLL transmembrane receptor which initiates a signaling cascade including the products of *TUBE* and *PELLE*, in the cytoplasm. CACTUS, an inhibitor protein, is then released from a complex with DORSAL. DORSAL is now free to be transported into the nucleus, where it can regulate the transcription of genes which lead to ventralisation. So although *DORSAL* is expressed in all parts of the egg, it is only active in the ventral region.

Similarly, *PHAN* is probably only required for the specification of dorsal identity in dorsal cells and not in ventral cells. At low temperature, PHAN is redundantly required for organ initiation. If the two functions of organ initiation and specification of dorsal identity were independent, both spatially and/or chronologically, then it would mean that PHAN must be interacting with two different sets of factors. One set of factors would be spatially restricted to dorsal cells and/or restricted to the timing of the specification of dorsal identity, while the other set would be restricted to the timing of organ initiation, if it is different to that of dorsal cell identity. *PHAN* may also be regulated in such a way that its expression in sepals, stamens and carpels is not required. A *PHAN* related gene has been isolated from *Antirrhinum* which is expressed in all floral whorls at the floritypic stage (Richard Waites pers. comm.). Perhaps this gene functions so that *PHAN* expression is redundant in whorls 1, 3 and 4.

The relatively recent tetraploidization events that occurred in plants suggests that plants may display a high level of genetic redundancy (Pickett and Meeks-Wagner, 1995). Gene duplication is an explanation for functional redundancy between

homologous genes. In *Arabidopsis*, *APETELA1* (*AP1*) and *CAULIFLOWER* (*CAL*) act redundantly to regulate floral meristem identity (Bowman *et al.*, 1993). *CAL* is completely redundant with *AP1* activity, while *AP1* has functions that are independent of *CAL*. Both have been shown to belong to the MADS box family of transcription factors, being the most close relatives of each other (Mandel *et al.*, 1992; Kempin *et al.*, 1995), so that their redundancy is probably a result of their similarity.

Gene duplication is an inadequate explanation for redundancy between nonhomologous genes. In these situations, it is probable that more than one gene of independent origins, converged in function during evolution. The production of flowers in *Arabidopsis* requires two overlapping pathways, one involving *AP1*, the other including *LEAFY* (*LFY*) (Bowman *et al.*, 1993; Shannon and Meeks-Wagner, 1993; Weigel *et al.*, 1992). Plants with single mutations of each gene still have some flower-like characteristics, thus, neither is absolutely required for the switch to a floral fate.. Epistasis experiments suggest that *AP1* and *LFY* act in partially redundant pathways, although they show no obvious homology to each other (Weigel *et al.*, 1992.; Bowman *et al.*, 1993). Transgenic experiments to consitutively express each gene (singly) showed that each can induce the formation of a flower by the main shoot and that *LFY* acts upstream of *AP1* (Weigel and nilsson, 1995; Mandel and Yanofsky, 1995). One of *LFY*'s functions may be to induce *AP1*, but both genes together are more efficient at promoting floral meristem identity than *LFY* on its own (Weigel and Nilsson, 1995; Mandel and Yanofsky, 1995). Wiegel and Nilsson (1995) found that the competence to respond to floral meristem identity genes is gained over time and that both *LFY* and *AP1* are required by younger meristems to acquire floral meristem identity. Eventually, competence increases till *LFY* alone is sufficient Thus, the control of floral fate in *Arabidopsis* provides a good example of both homologous and nonhomologous redundancies being involved in the same process.

Models explaining the evolutionary pressures that would maintain genetic redundancies have been proposed (Tautz, 1992; Thomas, 1993; Weintraub, 1993). These assume that mutations accumulate with one of the gene pair such that redundancies are eventually erased. So, only redundancies where each member has

pleiotropic as well as some independent functions, would experience positive selection to maintain the novel functions, so indirectly, the redundant function would endure. Conversely, negative selection prevents the accumulation of mutations, thus slowing gene divergence (Ohno *et al.*, 1968; Ohno, 1970; Hughes and Hughes, 1993). Therefore it is likely that the combatorial activities of positive and negative selection allows functional redundancies to be maintained. Divergence will occur at a faster rate if the products of the duplicated genes function alone rather than interacting with other proteins. Since negative selection would act against divergence of the biochemical functions of the genes, it is likely that divergence of regulatory elements would occur first, giving a novel expression pattern, after which, mutation of the biochemical function could follow. However, is also possible that transcription factors could have diverged to recognize different regulatory elements, thereby giving rise to a novel expression pattern of the biochemical function (Avila *et al.*, 1993). Duplication of transcription factors could therefore result in differential regulation of the biochemical function as seen in the case of MYB305 and MYB340 (Moyano *et al.*, 1996).

Genetic redundancies may obscure the true function of plant genes, so that analysis of the expression of all members of gene families is necessary. Plants homozygous for multiple mutations will facilitate the elucidation of the real 'complete loss of function' phenotype (Condie and Capecchi, 1994).

The *phan* phenotype is temperature sensitive

The timing and pattern of expression indicates that *PHAN* may play a role in the determination of lateral organ identity as being distinct from meristem and internode. A subset of determining lateral organ identity may be the specification of the dorsoventral axis. An additional feature of the *phan* mutant phenotype is its sensitivity to temperature. At 25°C, the *phan* mutant plants look very much like wild type. However, at 15°C the phenotype becomes more extreme, such that the meristem becomes quiescent.

There are many examples of mutations revealing the inherent temperature sensitivity of a pathway that overlaps with the function of the mutated gene. Strome *et al* (1995) screened for maternal-effect sterile (*mes*) mutants involved in normal germ line development of *C. elegans*. They identified the *mes-1* mutation which disrupts the generation of the primordial germ cell, early in the development of the embryo. They found that all ten of the *mes-1* mutant alleles were unusual in that they were temperature sensitive as well as being incompletely expressed. At the restrictive temperature of 25°C, homozygous mutant mothers produce a high percentage of sterile progeny, while at the permissive temperature of 16°C, they produced a low proportion of sterile progeny. Genetic and molecular evidence suggested that this was the null phenotype for *mes-1*, suggesting that the wild type gene takes part in an inherently temperature sensitive pathway. Temperature shift experiments were carried out to determine the temperature sensitive period, which went on to suggest that *mes-1* is involved in controlling the divisions that form the primordial cell. The partial expressivity of the alleles indicated that the pathway could function without the activity of *MES-1*, but to ensure its function at both low and high temperatures, active MES-1 is required. Strome *et al* suggested that MES-1 may function to stabilise a 'polarity component' which may otherwise become inactive, especially at high temperature.

In *Arabidopsis*, the *arrested development* (*add*) mutations show a temperature sensitive arrest in the development of seedlings (Pickett *et al.*, 1996). *add 1*, *2* and *3* show no specific temperature sensitive period, indicating that they are required throughout development. The temperature dependent variation in expressivity of the phenotype, as opposed to a switch from wild type to mutant phenotype suggests that the *add* mutations may reflect the temperature sensitivity of the biological process that they regulate. Although this is not necessarily the case, since the mutation could cause, for example, the expression of another protein in a region outside its normal domain, where it becomes unstable and sensitive to temperature. This would result in a temperature sensitive phenotype which does not reflect any inherent temperature sensitivity of the pathway, but temperature sensitivity caused by the mutation.

Therefore, at low temperatures, there is a requirement for *PHAN* expression in order to maintain the meristem. The sensitivity to temperature by initials in *phan* mutant apices, prior to emergence, corresponds to the timing of PHAN expression and lateral organ initiation. This suggests that *PHAN* expression is required in order to define lateral organ identity, and at low temperature, also acts to maintain the meristem. Since *PHAN* expression is confined to lateral organ initials and primordia, the effect on the meristem must be non cell-autonomous. All alleles of *phan* show the same temperature sensitivity, even the RNA null, *phan*-250G. This indicates that other factors which maintain the meristem are sensitive to temperature so that the lower the temperature, the greater the requirement for *PHAN*. *PHAN* and these other factors do not necessarily interact, *PHAN* merely performs the same task. The temperature shift experiments reveal that the process which maintains an active meristem is temperature sensitive. At high temperature, the activity of *PHAN* is not required and the other components of the pathway are sufficient. However, at lower temperatures, these other factors are inhibited, possibly due to the inability of the protein products to function at low temperatures, such that functional PHAN is required. In contrast to many temperature sensitive mutations, low temperature is restictive for the *phan* phenotype. It is usual for the restrictive temperature to be high in other cases due to protein denaturation at high temperature. It is likely that in the case of the *phan* phenotype, low temperature results in kinetic problems or proteins being unable to

perform necessary conformational changes. *phan* mutant plants look wild type at 25°C indicating that all aspects of *PHAN* function are genetically redundant. So what are the other members of the process in which *PHAN* is a redundant member?

Other genes involved in meristem function

STM is required for the formation of the embryonic SAM and is also required for its maintenance postembryonically. *STM* is expressed in the undifferentiated meristem cells and is down regulated as cells become differentiated. The *Antirrhinum* homologue of *STM* is expressed similarly in lateral organ initials. The loss of *STM* expression acts as the earliest marker for lateral organ fate. Since *PHAN* and *AmSTM* have complementary expression patterns, it was thought that the expression of *PHAN* in initials may cause *AmSTM* to be limited to undifferentiated cells. However, it has been demonstrated that *PHAN* does not regulate the expression pattern of *AmSTM*, since *AmSTM* is expressed in a normal pattern, though at a reduced level, in *phan*-250G null mutants. It is still to be determined if *PHAN* expression is confined to initials due to the action of *AmSTM*. It is possible that *PHAN* represses the expression of *STM* in organ initials and primordia, but upregulates the expression of *STM* in neighbouring undifferentiated cells of the meristem to a critical level, via signalling across the boundary. This fits in with the hypothesis that *PHAN* regulates the expression of *STM* in the adjacent domain. Both *PHAN* and *AmSTM* expression is absent from arrested meristems, so that they both act as markers for functional meristems.

The *NAM* gene of Petunia is also required for SAM formation. Expression is seen at a later stage of embryogenesis than is *STM*, where it forms a circle around the embryonic SAM. Postembryonically, *NAM* continues to be expressed in rings encircling organ and meristem primordia. *FIM* and it's orthologue *UNUSUAL FLORAL ORGAN* (*UFO*) (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995) are expressed similarly to form a circle around the SAM and postembryonically to create boundaries between meristem and organ initial cells. It is likely that these genes that express at the boundaries function to define functional domains, for

example, to suppress any further organ initiation or cell growth in boundary regions, so keeping organ domains distinct. They could also function to facilitate signalling between domains such that, say, *PHAN* could down regulate the expression of *STM* in organ initial domains, whilst up regulating its expression outside this domain.

Organogenesis from the SAM

A working model for the formation of lateral organs from the *Antirrhinum* SAM proposed that while *PHAN* is expressed in organ initial and primordial cells to specify lateral organ identity, *AmSTM* is expressed in the surrounding undifferentiated cells of the SAM, with *NAM* like genes expressed at the boundaries of these domains. The expression patterns of *PHAN* and *AmSTM* were found to be complementary, as predicted, however, a *NAM* like gene was not identified. Interestingly, mutations affecting meristem function, also resulted in aberrant lateral organ formation, suggesting that the two processes are closely related. An example is the temperature sensitive *add1* mutation (Pickett *et al.*, 1996) which results in the production of one or two true leaves and then halts further development, at the restrictive temperature. Following germination, lack of *ADD1* product at the restrictive temperature results in the formation of abnormal leaves which show a loss of dorsoventrality and is followed by arrest of the meristem.

The model proposes that genes expressed in all three domains (initials/primordia, undifferentiated meristem and boundaries between them) act non cell-autonomously to ensure specification of lateral organs and meristem function. Perhaps loss of expression of one, would lead to the misexpression of another. While it has been shown that loss of *PHAN* expression does not change the pattern of *AmSTM* expression (although it may lower the level of expression), the effects of the loss of *AmSTM* and an *Antirrhinum NAM*-like gene have still to be investigated.

Surgical experiments and mutants which disrupt meristem function have suggested that there is a requirement for a functional meristem for normal leaf development, since the leaves produced in both cases have abnormal morphologies, lacking in

dorsoventral characteristics. However, there have been no reports of mutations which abolish leaf initiation leaving a stem only. This raises the possibility that conversely, organ specification may be required for a functional meristem. Leaves lacking dorsoventrality have been reported but normal leaves also show proximodistal differences. Perhaps defining a leaf primordium as separate from the rest of the meristem involves the specification of both these axes. At intermediate temperature *phan* mutants lack a dorsoventral axis and at low temperature, there is a loss of the proximodistal axis and meristem arrest. Therefore *PHAN* may define lateral organ identity by playing a role in both processes, as revealed by their differential sensitivity to temperature in *phan* mutants.

Leaves show differences along their dorsoventral and proximodistal axes. However, leaves are also expanded symmetrically about their midvein. Mutation of the *ARGONAUTE* (*AGO1*) gene of *Arabidopsis* leads to the loss of lateral expansion, a progressive loss of dorsoventral differences and reduction of differences along the proximodistal axis (Bohmert *et al.*, 1998). The Waites and Hudson model (1995) suggests that lateral growth is a result of the interaction at the boundary of dorsal and ventral domains in the primordia. The *ago1* phenotype is consistent with this model since the loss of lateral growth is accompanied by a loss of dorsoventrality. Over expression of *AGO1* resulted in an excess of lateral expansion and the formation of shoot like structures on leaves, indicating that ectopic meristems may have formed (Bohmert *et al.*, 1998). *AGO1* seems to affect all three axes of growth. Perhaps the over expression of *AGO1* caused the ectopic specification of these axes of growth which is in turn, sufficient to determine ectopic leaf formation. This indicates that specifying the dorsoventral axis (which results in lateral growth) and the proximodistal axis may be sufficient to define a leaf. It is possible that ectopic definition of lateral organs is sufficient to prompt the formation of ectopic meristems. That is, the definition of a SAM is a group of cells that initiates lateral organs.

Another gene which affects axis formation, is *EMB30* from *Arabidopsis* (Shevell *et al.*, 1994). Mutations of this locus result in seedlings with aberrant fused cotyledons and disrupted shoot development such that weaker mutations produce shoot like

growths, but never normal stems or flowers. The production of roots is abolished except for occasional root hair like structures. The phenotype can be explained by the failure of the apical basal axis to be correctly specified during embryogenesis due to the asymmetrical division at the first zygotic being symmetrical instead. This asymmetric division would normally give rise to a smaller apical cell which forms the embryo, and a larger basal cell which will form the root meristem and suspensor. This disruption of apical-basal polarity results in a deviated plane of cell division, plane of cell expansion and the rate of cell division, all of which determine plant shape. The EMB30 protein shows homology to the SEC7 protein of yeast which is involved in protein transport in the secretory pathway (Esmon *et al.*, 1981), which would indicate that EMB30 plays a general cellular function (Shevell *et al.*, 1994). However, Busch *et al* (1996) found that *EMB30* (also known as *GNOM*) was more similar to the yeast *YEC2* gene which is not essential for cell function, similarly to *GNOM*. The partial complementation by *gnom* alleles has suggested that the GNOM protein may be homomeric and consist of identical subunits. Thus the fact that *GNOM* is expressed constitutively means that it is possible that the physical association of the GNOM subunits is necessary for the activation of the protein, similarly to the TOLL protein (Busch *et al.*, 1996; Schneider *et al.*, 1991), thereby interpreting a pre-pattern.

Therefore a reduction of apical-basal polarity in *emb30/gnom* results in a disruption of both dorsoventral and proximodistal polarity. It is possible that the apical-basal axis needs to be set to allow the definition of the dorsoventral and proximodistal axes in reference to it.

Leaves from *Antirrhinum* and maize can be considered to be analogous structures and homologous genes have been identified. The *ROUGH SHEATH 2* (*RS2*) gene from maize has been shown to be the likely homologue of *PHAN* and is expressed similarly to *PHAN* in lateral organ primordia, but from a much later stage following emergence from the meristem (J. Langdale. pers. comm). Recessive alleles of *rs2* show disorganisation of the growth and differentiation of the blade-sheath boundary and narrow leaves. Severe phenotypes result in certain genetic backgrounds and are sensitive to environmental factors. These include semi bladeless and leaves lacking a midrib. Narrow thread like leaves are also seen. However, unlike the needle like leaves seen in *phan* plants, these have dorsoventral asymmetry. It has been shown that leaves of *rs2* mutants ectopically express five *knox* (*KNOTTED*-like homeobox) genes, showing that *RS2* negatively regulates their expression in leaves (Schneeberger *et al.*, 1998). In *Antirrhinum*, it has still to be confirmed that *PHAN* regulates the level of *STM* expression, and if it regulates the pattern and/or level of expression of other *KN1*-like genes. It is probable, due to the high level of redundancy in maize, that maize has other *PHAN* like genes, one of which may be functionally more similar to *PHAN* than is *RS2*.

Therefore, it seems that in general, homologous genes from *Antirrhinum*, *Arabidopsis* and maize may be involved in a similar way to regulate leaf development. Perhaps *PHAN* was present in the ancestral leaf and was instrumental in bringing about the lateral expansion of the lamina to produce an effective solar panel. At an earlier stage, it is possible that *PHAN* was present to set up a dorsoventral axis and interacted with a gene that set up a proximodistal axis, resulting in growth in a novel axis lateral to the stem, to give a lateral organ.

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The *PHANTASTICA* Gene Encodes a MYB Transcription Factor Involved in Growth and Dorsoventrality of Lateral Organs in *Antirrhinum*

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Summary

The organs of a higher plant show two fundamental axes of asymmetry: proximodistal and dorsoventral. Dorsoventrality in leaves, bracts, and petal lobes of *Antirrhinum majus* requires activity of the *PHANTASTICA* (*PHAN*) gene. Conditional mutants revealed that *PHAN* is also required for earlier elaboration of the proximodistal axis. *PHAN* was isolated and shown to encode a MYB transcription factor homolog. *PHAN* mRNA is first detected in organ initials before primordium initiation. The structure and expression pattern of *PHAN*, together with its requirement in two key features of organ development, are consistent with a role in specifying lateral organ identity as distinct from that of the stem or meristem. *PHAN* also appears to maintain meristem activity in a non-cell-autonomous manner.

Introduction

The above-ground part of a flowering plant consists of a series of organs born on continuous stem axes. A plant produces several different organ types in a defined sequence during its lifetime—leaves during vegetative growth and various floral organs later in development. This production of repeated structures with different identities can be compared to the segmentation pattern of insects. In *Drosophila*, different combinations of segment identity gene functions specify differences between segments, while shared segment polarity gene functions are responsible for similarities between them. Unlike plant organs, however, the segments of *Drosophila* arise synchronously by subdivision of the embryo rather than sequentially. In plants, much is known about genes involved in establishing the differences between organs (reviewed by Weigel and Meyerowitz, 1994), whereas little is known of the basis of similarities between organs.

The chief similarity between the organs of a higher plant is that they show two major axes of asymmetry. First, they exhibit a proximodistal axis lateral to the stem and are therefore collectively termed lateral organs (although organs may appear to form directly from the apex of the floral meristem as with carpel development in some species). The proximodistal axis becomes apparent as groups of organ initial cells on the flanks of the meristem form a primordium with a novel direction of growth. Morphological differences may be elaborated later along this proximodistal axis, for example, between the broad distal leaf blade and the narrower petiole (stalk) proximal to the stem. Second, lateral organs typically show dorsoventral asymmetry. Most are flattened in a plane perpendicular to their dorsoventral axis. The developmental stage at which this flattening becomes apparent varies between species. In many monocots, such as maize, it can be first seen when primordia emerge from the apical meristem, whereas in other species, proliferation may lead to flattening only after primordial emergence (e.g., Poethig and Sussex, 1985). Dorsoventral asymmetry is further apparent in the pattern of cell types within organs. In most organs, the upper (adaxial) surface is often distinct from the lower (abaxial), and dorsoventral differences may also be seen in internal tissues. In contrast to lateral organs, the main stems of most flowering plants are radially symmetrical.

One working hypothesis is that the two axes of lateral organs—proximodistal and dorsoventral—may be the result of genetic functions that are common to all organs. Because these characteristics are apparent from early in organ development, it is likely that the corresponding genetic functions are expressed in groups of initial cells that have assumed lateral organ identity at, or before, the time that they are visible as primordia.

What commits meristem cells to a lateral organ fate and therefore to elaboration of proximodistal and dorsoventral axes remains poorly understood. At least two genes required for normal meristem function are expressed in domains which suggest that they are involved in a meristematic prepattern distinguishing organ initials from the remainder of the shoot apical meristem (SAM). The homeobox gene, *SHOOT MERISTEMLESS* (*STM*), is required for the formation and maintenance of the SAM in *Arabidopsis* and is transcribed in cells of the apical meristem and internode initials (Clark et al., 1996; Long et al., 1996). Absence of *STM* expression from groups of cells flanking the SAM is the earliest known marker for lateral organ initials. In Petunia, the *No apical meristem* gene is expressed around the boundary between lateral organ initials and the SAM and is required for SAM formation (Souer et al., 1996). However, no gene has yet been identified that shows expression confined to the initials of all lateral organs and could therefore be a potential determinant of lateral organ identity.

We have previously identified a requirement for the *PHANTASTICA* (*PHAN*) gene in one lateral organ characteristic—dorsoventrality—in *Antirrhinum* (Waites and Hudson, 1995). In phan mutants, tissues associated with the dorsal part of the wild-type leaf can be replaced by tissues with ventral characteristics, suggesting that *PHAN* is required for identity of dorsal leaf initial cells. A relationship between *PHAN*-dependent dorsal cell identity and flattening of the leaf blade was also suggested, based on the appearance of phan mutant leaves that were mosaics of dorsal and ventral tissues. In all

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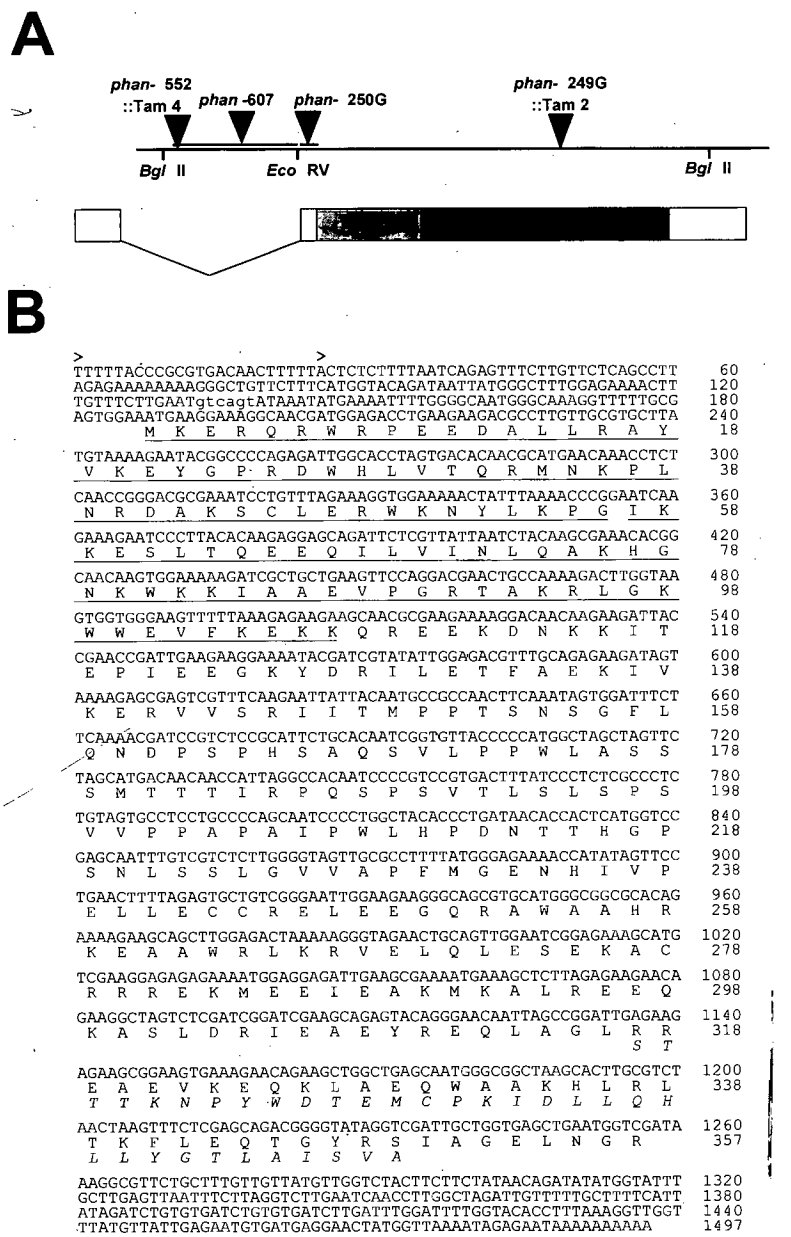

Figure 1. Structure of the *PHAN* Locus

(A) Map of the *PHAN* genomic region. The insertion sites of *Tam4* in *phan*-552 and of *Tam2* in *phan*-249G are shown by black triangles (not to scale) on the restriction map. The regions in which *phan*-607 and *phan*-250G carry insertions are represented by lines with triangles above the map. Boxes below the restriction map represent exons. White boxes denote untranslated exon sequences, and the stippled box, the region encoding the conserved MYB domain.

(B) The amino acid sequence of PHAN is shown below its nucleotide sequence. The 5′ end of the cDNA sequence was obtained by RACE and is therefore assumed to represent the longest transcript. A second transcription initiation site at position 25 is indicated by an arrowhead. Splicing of an intron in the 5′UTR can occur at one of two donor sites, separated by the sequence shown in lower case. The two MYB repeats of the PHAN protein are underlined, and the novel C-terminal region encoded by the *phan*-249G allele, as the result of *Tam2* insertion, is shown in italics below the wild-type amino acid sequence.

cases, outgrowths resembling ectopic leaf blades were formed at the junctions between dorsal and ectopic ventral tissues. This indicated that interaction between dorsal and ventral cells might be responsible for outgrowth of the leaf blade in wild type in an axis perpendicular to the proximodistal axis of the primordium. *PHAN* also appeared to be required for dorsoventrality of bracts and petal lobes, but not for normal development of other lateral organs.

To examine further the function of *PHAN* in lateral organ development, we exploited the cold sensitivity of the phan mutant phenotype. This revealed an additional requirement for *PHAN* in proximodistal growth and patterning of lateral organ primordia. We have also isolated *PHAN* and shown that it encodes a MYB-related transcription factor. Expression of *PHAN* is specific to the initials and early primordia of all lateral organs. It is expressed throughout each primordium, indicating that spatially specific expression of *PHAN* within the primordium is not responsible for the establishment of proximodistal or dorsoventral axes. Rather, *PHAN* may interact with other genes that have spatially restricted expression patterns. The biochemical function, expression pattern, and requirement for *PHAN* in elaboration of proximodistal and dorsoventral organ axes are consistent with a role in the determination of lateral organ identity as distinct from that of the meristem and stem. We have also shown a requirement for *PHAN* in maintaining the activity of apical stem cell populations, which suggests that determination of lateral organ identity is essential for function of the apical meristem in higher plants.

Results

Isolation of the *PHANTASTICA* Gene

The *PHANTASTICA* (*PHAN*) locus was defined by four recessive mutations that disrupted development of leaves, bracts, and petal lobes. One mutation, *phan*-607, arose in a transposon mutagenesis program (Carpenter and Coen, 1990) and was shown to be allelic with two classical mutations, *phan*-249G and *phan*-250G (Baur, 1926; Stubbe, 1932). Plants homozygous for *phan*-607 gave rise to one wild-type plant among ~500 mutant progeny, suggesting that the mutation was caused by a transposon which could excise from the locus at low frequency to restore a functional *PHAN* allele. A further mutant allele, *phan*-552, was identified in a targeted transposon mutagenesis experiment (Waites and Hudson, 1995) and exploited to clone the locus. The mutation had been obtained by crossing homozygous *phan*-249G mutants to the wild-type line JI.75, which carries active transposons in its genetic background. A single phan mutant was identified among ~18,000 wild-type F1 progeny and assumed to be heterozygous for *phan*-249G and a newly mutated allele, *phan*-552. Southern hybridization revealed a copy of the *Tam4* transposon in the *phan*-552/*phan*-249G mutant, as a 6.3 kb BglII fragment that was not present in eight of its wild-type siblings. To test whether this copy of *Tam4* was responsible for the *phan*-552 mutation, the mutant was back-crossed to its *phan*-249G parent. A small proportion of the progeny (42 plants in 13 independent families) had wild-type phenotype, indicating that they carried *PHAN*$^+$ revertant alleles. About 50% of the mutant progeny were found to have inherited the copy of *Tam4*—a frequency expected from segregation of the *phan*-552 allele in gametes of the *phan*-552/*phan*-249G parent—whereas all 42 of their PHAN$^+$ revertant siblings lacked it. These results suggested that the *Tam4* insertion was responsible for the *phan*-552 mutation. The copy of *Tam4* and sequences flanking it were cloned (see the Experimental Procedures), and sequence analysis revealed that *Tam4* had inserted near to the end of a wild-type BglII fragment of 1.6 kb. Plants carrying the three other *phan* mutations also showed polymorphisms in this fragment consistent with different DNA insertions (Figure 1A). In the case of *phan*-607, transposon excision was correlated with phenotypic reversion to wild type. Together, these results strongly suggested that at least part of the *PHAN* gene was contained within the cloned DNA. The sequence flanking *Tam4* contained a single long open reading frame, and comparisons of wild-type genomic and cDNA clones indicated that this represented the entire PHAN protein coding region. An intron was present within the 5′ untranslated region, which began at one of two splice donor sequences 192 or 198 bp downstream of the major transcript initiation site and ended 49 bp upstream of the translation initiation codon (Figure 1B). The *Tam4* transposon in *phan*-552 was therefore present within this intron, 351 bp upstream of the splice acceptor.

The Structures of *phan* Alleles Correlate with Their Phenotypic Severity

The four *phan* mutations differed in their effects on petal development. The petal lobes of *phan*-250G mutants were consistently reduced to ventralized needles whereas the other *phan* mutations allowed development of petal lobes to increasing degrees, in the order *phan*-250G < *phan*-249G < *phan*-607 < *phan*-552. Weaker mutations were found to be dominant to stronger ones, suggesting that the weak and intermediate mutant alleles conferred reduced *PHAN* activity (hypomorphs). The structure and expression of the mutant alleles was therefore characterized further.

The weakest alleles, *phan*-607 and *phan*-552, contained insertions within the intron and were able to encode *PHAN* mRNA of wild-type size at reduced abundance (data not shown), suggesting that transposon sequences could be removed by splicing of the intron to allow production of a functional transcript. The intermediate allele, *phan*-249G, was found to contain a stable insertion of the *Tam2* transposon within the *PHAN* coding region (Figure 1A), which was flanked at its upstream end by an additional 2 bp (TC), suggestive of an aberrant insertion or abortive transposition event involving only this end of *Tam2*. As a result, the *phan*-249G allele had the potential to encode a novel protein in which the 40 C-terminal residues of PHAN were replaced by 34 amino acids encoded by *Tam2* (Figure 1B). Because the *phan*-249G allele conditioned an intermediate mutant phenotype, the novel protein was assumed to be expressed and to be at least partially functional. The strongest mutant allele, *phan*-250G, contained a stable insertion of about 4.5 kb between the *PHAN* splice acceptor site and start of translation (Figure 1A). No *PHAN* mRNA could be detected in vegetative or inflorescence tissues of plants homozygous for this mutation (Figure 3E), suggesting that the insertion prevented production of a functional *PHAN* transcript and therefore that *phan*-250G was a null allele.

The four *phan* mutations conditioned nearly identical vegetative phenotypes. The leaves produced early in development typically had laminae with patches of ectopic ventral tissues. Late leaves and bracts were usually reduced to ventralized needles, and intermediate leaves were either narrower than wild type, with laminae arising from more dorsal positions, or mosaics of normal and ventralized tissues. Although all the *phan* mutant alleles contained transposons or DNA insertions suggestive of transposons, over 50,000 progeny from each of the *phan*-249G and *phan*-250G mutants contained no wild-type revertants, suggesting that the mutations in these lines were genetically stable. Therefore, the variability in mutant leaf morphology did not appear likely to result from restoration of *PHAN* activity on somatic transposon excision; nor could the variability be attributed to residual *PHAN* activity in mutants because it was shown by plants homozygous for the potential null allele, *phan*-250G. It therefore seemed likely to reflect the activity of independently expressed factors that could partially substitute for *PHAN* in developing leaves.

PHAN Encodes a MYB-Related Transcription Factor

PHAN had the potential to encode a 42.4 kDa protein of 357 amino acids (Figure 1B). Imperfect tandem repeats of 56 and 51 amino acids at the N terminus of the PHAN protein showed significant similarity to the motifs

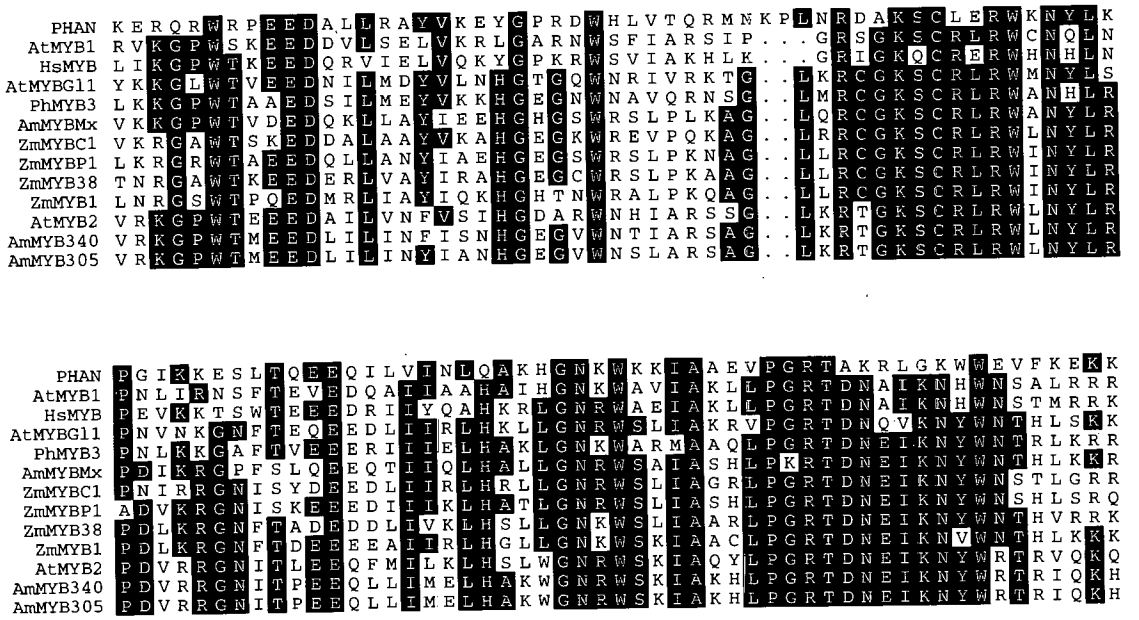

Figure 2. Sequences Similar to PHAN

The two imperfect amino acid repeats of PHAN are shown aligned with those of a number of MYB transcription factors that have defined functions and/or demonstrated DNA binding activities. Residues that are identical in the majority of these proteins are boxed. The nomenclature for MYB proteins is that of Martin and Paz Ares (1997).

present in 1–3 copies close to the N termini of all known MYB-related transcription factors (Figure 2). Although PHAN contained two of these repeats, in common with most plant MYB proteins, it showed a high degree of similarity to the product of only one gene, of unknown function, induced on root-knot nematode infection of tomato (Bird and Wilson, 1994). It differed from other MYB proteins in three obvious respects: the C-terminal region of the second repeat (residues 93–102) showed little sequence conservation, PHAN had only one amino acid upstream of the first repeat, and the first repeat was 2 or 3 amino acids longer than that of other MYB proteins (Figure 2).

Downstream of the conserved MYB domain, PHAN showed no sequence similarity to any characterized protein, except for the product of a related gene of unknown function from tomato. However, in common with many MYB proteins, the C-terminal region contained a high proportion of negatively charged residues.

PHAN mRNA Is Restricted to Lateral Organ Initials and Primordia

Expression of *PHAN* was examined in wild-type seedlings. In the early phase of vegetative growth, wild-type plants produce a pair of leaves at each node from groups of initials at opposite sides of the shoot apical meristem (SAM) and at ~90° to the pair of leaf primordia at the previous node (decussate phyllotaxy). By convention, nodes with emerged leaves are numbered from the top of the shoot downward, so that the pair of primordia to have emerged most recently from the apex are termed P_1. Cells within the SAM that will form the next pair of primordia are designated P_0, and initials of subsequent primordia, I_1, I_2, and so forth. The primordia of *phan* mutant leaves differed from those of wild type from about the P_1 stage, by their more radial appearance, indicating that *PHAN* is required for dorsoventrality early in leaf development (Waites and Hudson, 1995).

Digoxigenin-labeled antisense RNA was transcribed from part of the *PHAN* gene downstream of the conserved *MYB* region and used to probe sections of wild-type seedlings. *PHAN* mRNA expression was detected in two opposite domains of the meristem corresponding to the positions of P_0 initials and in newly emerged P_1 primordia (Figures 3A and 3B). To examine whether *PHAN* mRNA was restricted to leaf initial cells, its domain of expression was compared to that of *AmSTM*, the *Antirrhinum* homolog of the *Arabidopsis SHOOT MERISTEMLESS* gene (see the Experimental Procedures). In *Arabidopsis*, *STM* is expressed in cells of the SAM, but not in leaf primordia, and loss of expression from leaf initials provides the earliest known marker of leaf fate (Long et al., 1996). In adjacent sections of the wild-type *Antirrhinum* SAM, the domains of *PHAN* and *AmSTM* expression appeared to be complementary (Figures 3C and 3D). *PHAN* mRNA was restricted to leaf initial cells and uniformly distributed throughout them. *PHAN* mRNA expression persisted in developing leaves but was undetectable after late stage P_3. By this stage, the wild-type leaf showed dorsoventral differences in cell type (e.g., trichomes produced from only the dorsal surface) and marked dorsoventral flattening (Figures 3A and 3B).

Because *PHAN* was also required for normal development of bracts and petal lobes, its expression was characterized in wild-type inflorescences. In contrast to young vegetative shoots, the inflorescence produces

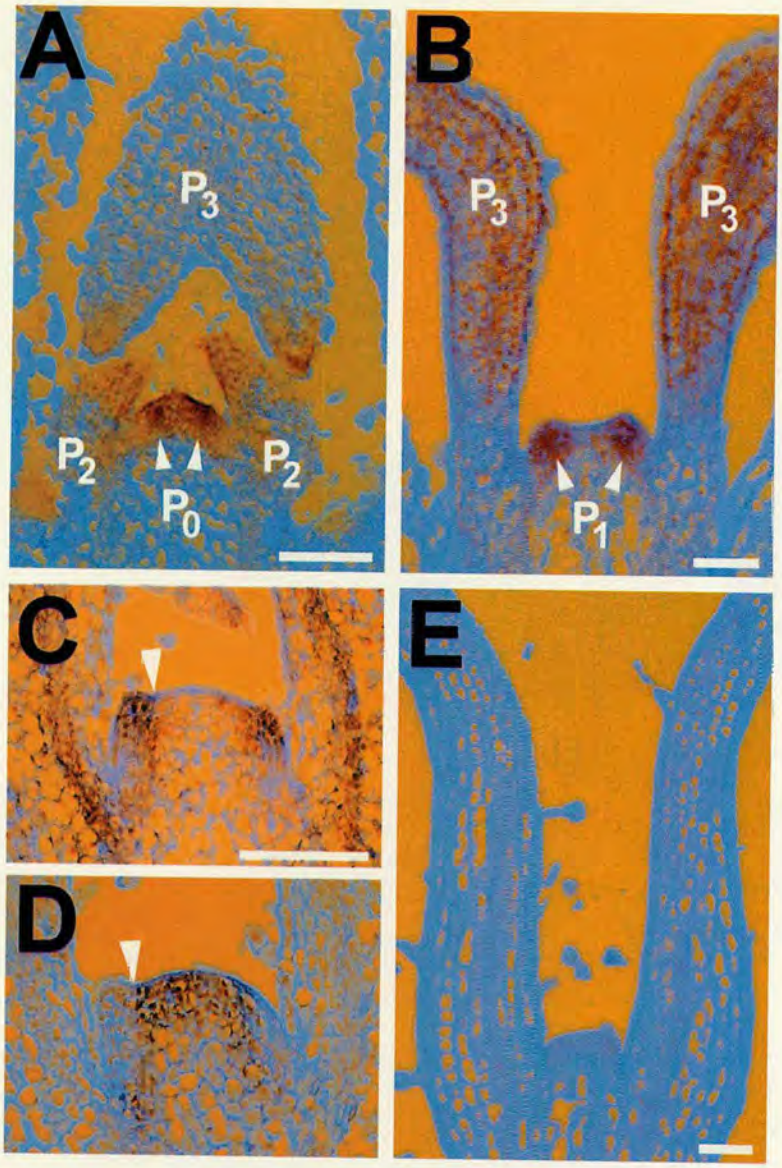

Figure 3. Vegetative Apices Probed with *PHAN* or *AmSTM*

Longitudinal sections of wild-type shoot tips passing through leaf initials and primordia at different developmental stages (A and B) were probed with *PHAN*. Hybridization of adjacent sections of wild-type apices with *PHAN* (C) or *AmSTM* (D) probes revealed that their expression patterns were complementary (see arrowheads). No *PHAN* mRNA could be detected in apices of the *phan*-250G mutant (E). Scale bar is 100 μm. P_0, etc., denotes the developmental ages of leaf initials and primordia (see text for details).

bracts singly in a spiral arrangement, so that each bract primordium emerges from the apex above P_4 and P_6 primordia initiated earlier. *PHAN* mRNA was detected in domains of the inflorescence meristem corresponding to the position of bract initials from stage I_3 onward (Figure 4A). Its expression was compared with that of *FLORICAULA* (*FLO*), which is first detected in bract initials from the P_0 stage, providing an early marker for bract fate, and subsequently in floral meristems subtended by existing bract primordia (Coen et al., 1990). Hybridization to adjacent sections showed that *PHAN* expression began earlier than that of *FLO* (Figure 4B) and revealed that the domain of *PHAN* mRNA expression in older, P_0, bract initials coincided with that of *FLO* (data not shown). As in leaves, *PHAN* expression persisted in developing bracts becoming undetectable by about stage P_{10}.

During development of wild-type flowers, *PHAN* mRNA was initially absent from floral meristem precursors within the inflorescence meristem and from newly initiated floral meristems, marked by uniform expression of *FLO* (Figures 4A and 4B). It was subsequently detected in sepal initials slightly before the stage that *FLO* expression became limited to the same domain (Figures 4C and 4D). At about the floritypic stage, when expression of the floral homeotic genes *DEFICIENS* (*DEF*) and *PLENA* (*PLE*) became established in partially overlapping domains of the floral meristem, *PHAN* mRNA declined slightly in sepal primordia and appeared in petal initials that are marked by expression of *FLO* but not *PLE* and in stamen initials that express *PLE* but not *FLO* (Figures 4E–4F). The timing of floral homeotic gene expression has suggested that petal and stamen identities are determined at approximately the same time (Bradley et al., 1993), and primordia of both organ types initiate almost simultaneously (Carpenter et al., 1995). In flowers at later stages of development, *PHAN* expression was detected in stamen and carpel initials and

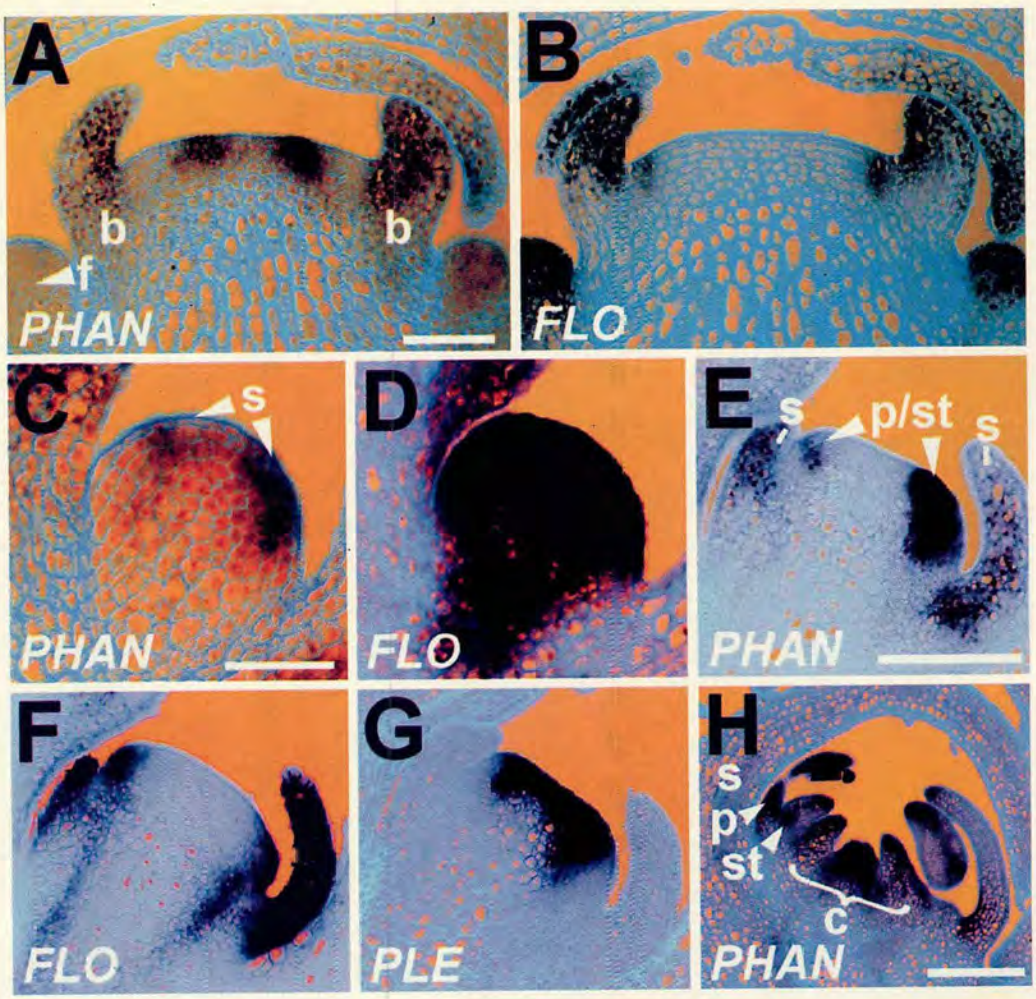

Figure 4. *PHAN* mRNA Expression in Inflorescence and Floral Meristems

Adjacent longitudinal sections of wild-type inflorescence apices were probed with (A) *phan* or (B) *FLO*. In serial sections of early floral meristems, *PHAN* mRNA was detected in sepal initials (C) before expression of *FLO* became confined to the same domain (D). The pattern of *PHAN* transcription in wild-type floral meristems at about the floritypic stage (E) was compared in serial sections to that of *FLO* (F) and *PLE* (G) and characterized in wild-type flowers at a later stage in development (H). Scale bars represent 100 μm in (A), (E), and (H) and 50 μm in (C). Young floral meristems are indicated by f, and the initials or primordia of bracts, sepals, petals, stamens, or carpels are indicated by b, s, p, st, and c, respectively.

primordia (Figure 4H). *PHAN* therefore appeared to show an equivalent pattern of expression in all lateral organs, although phan mutant phenotypes indicated that it was required only for development of normal leaves, bracts, and petal lobes.

PHAN Is Needed for Lateral Organ Initiation and Meristem Function

The phan mutant phenotype was almost completely suppressed in plants grown at 25°C, and enhanced at lower temperatures. At 17°C, all except the first two pairs of leaves were completely ventralized. Because this temperature response was shown by all mutants, including those carrying the likely null allele *phan*-250G, it was assumed to reflect the temperature sensitivity of functions that reduced the requirement for *PHAN* at higher temperatures. We therefore tested whether a greater requirement for *PHAN* might be imposed by growing plants at an even lower temperature. At 15°C, *phan* mutant seedlings arrested development after producing only one or two pairs of leaves (Figure 5A). Primordia of the subsequent pair of leaves either failed to initiate or arrested development at the P_1 stage (Figure 5B). The apical meristems of these plants also ceased growth and appeared flatter and less organized that those of wild-type plants (compare Figures 5B and 5C). Meristems that had formed in the axils of existing leaves were affected in a similar way. In contrast, wild-type plants were able to grow normally and to flower at 15°C. (Figure 5D). These results indicated that *PHAN* had additional roles in initiation and proximodistal outgrowth of leaf primordia, and in maintaining activity of shoot apical meristems. Reducing the requirement for *PHAN*, by returning plants to the permissive temperature, allowed leaf initiation and meristematic growth to resume even after 6 weeks at 15°C (Figure 5E). Primordia initiated upon shifting to the permissive temperature formed normally shaped leaves, although these were irregularly

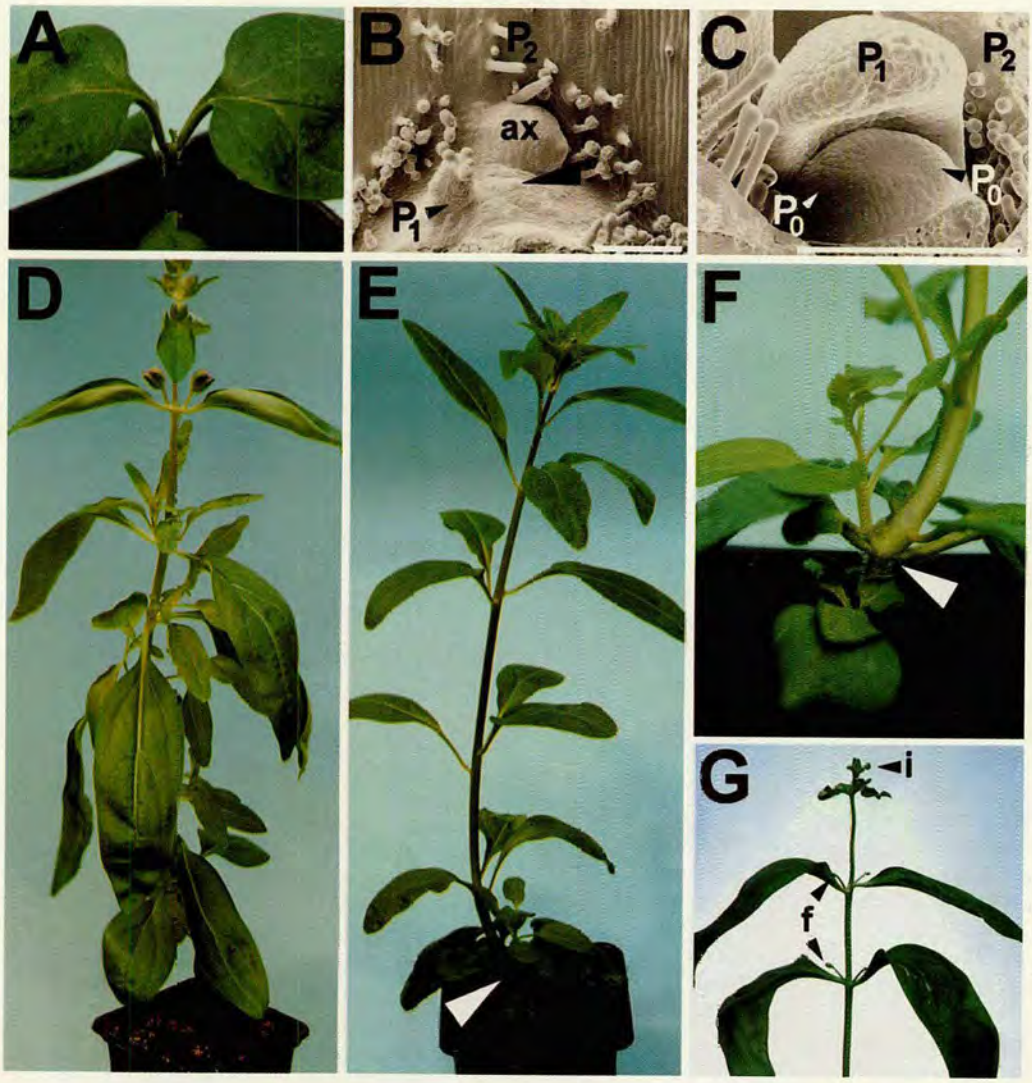

Figure 5. Temperature-Sensitive Effects of *phan* Mutations on Organ Initiation and Meristem Function

(A) A *phan*-607 mutant seedling arrested in growth after germination at the nonpermissive temperature of 15°C. (B) The apical region of this plant compared to the apex (C) of a wild-type plant (D) of a similar age under the same conditions. A secondary meristem in a leaf axil is indicated by ax, and the arrowhead points to the region corresponding in position to the apical meristem of wild type. Shifting *phan* mutants from 15°C to the permissive temperature of 25°C allowed meristem function to resume (E), although initial leaves were aberrantly positioned on the stem axis (F). In this case, the major shoot produced upon shifting to the permissive temperature (above the white arrowheads) arose from an arrested axillary meristem; in other cases, it was produced by a resumption in growth of the SAM. Shifting plants to the nonpermissive temperature later in development (G) inhibited inflorescence (i) and floral meristem (f) development and initiation and growth of floral organ primordia. Scale bars denote 100 μm.

spaced on the stem axis for several nodes (Figure 5F), suggesting an involvement of *PHAN* in phyllotaxy.

To investigate when *PHAN* was required for leaf initiation, phan mutants were grown initially at 25°C and then transferred to the restrictive temperature of 15°C. The developmental stage of each plant at the time of transfer was estimated from the length of existing leaves (see the Experimental Procedures). In a significant proportion of plants (61%), the last leaves to be produced were those derived from P_1 primordia (15%) or P_0 initials (46%) at the time of the temperature shift. The requirement for *PHAN* in the emergence of these primordia was therefore consistent with the earliest observed stages of *PHAN* mRNA expression. The remaining 39% of plants showed a more delayed response to the temperature shift, initiating an additional one or two pairs of leaves before meristem arrest. Ventralized leaves were observed only rarely, and leaves that had been older than stage P_1 at the time of the temperature shift were almost always able to develop normally. Similarly, phan mutant meristems that had arrested at the restrictive temperature initiated only dorsoventrally flattened leaves on return to 25°C. This suggested that the requirement for *PHAN* in dorsoventrality did not extend beyond that in primordium emergence and early proximodistal growth.

The potential role of *PHAN* expression in bract and floral organ primordia was examined by transferring phan mutants to the restrictive temperature after the

transition to flowering. Initiation of bract primordia and activity of inflorescence meristems were inhibited. Furthermore, floral meristems either failed to initiate organ primordia or arrested after production of one or more whorls of rudimentary floral organs (Figure 5G). These results suggested that there was a similar requirement for *PHAN* in initiation of all lateral organ primordia and in maintaining the normal activity of all apical meristems. The effects of the temperature shift on floral development occurred several nodes below those on bract initiation, consistent with the delay of about six nodes between initiation of a bract primordium and initiation of the floral organs from the meristem in the axil of that bract (Carpenter et al., 1995).

Discussion

We have demonstrated that *PHAN* is required for elaboration of the proximodistal axis of all lateral organs, for dorsoventral asymmetry in a subset of organs, and to maintain the activity of apical meristems. The *PHAN* gene encodes a MYB-related transcription factor. MYB proteins are characterized by 1–3 N-terminal copies of a conserved sequence (the MYB repeat) that function in binding target DNA (Tanikawa et al., 1993), and C-terminal terminal regions that show little sequence conservation. Higher plant genomes encode numerous MYB proteins, and those for which functions have been determined regulate diverse developmental and metabolic processes (Martin and Paz Ares, 1997). The PHAN protein contains two repeats, in common with most plant MYBs. However, these are unconventional in several respects. The first repeat is 2 or 3 amino acids longer than other MYB proteins and has only one amino acid upstream of it, rather than a short leader sequence. The C-terminal region of the second repeat, which is highly conserved in other proteins and implicated in DNA binding specificity, is poorly conserved in PHAN. The *Antirrhinum* genome has the capacity to encode at least one additional PHAN-like protein, which shares these unconventional characters and also shows conservation with PHAN in its C-terminal region (unpublished data). These results suggest that *PHAN* is a member of a small subfamily of *MYB* genes which may be the result of an ancient divergence. The only other characterized member of this subfamily is expressed following root-knot nematode infection of tomato roots (Bird and Wilson, 1994). Infection induces differentiation of a novel cell type and ectopic expression of a number of genes (Opperman and Conkling, 1994), and the functional significance of *PHAN*-like gene expression in this process therefore remains unclear.

PHAN Is Required for Elaboration of a Proximodistal Axis in All Lateral Organs

The phan mutant phenotype is more severe at lower temperatures. This cold sensitivity is shared by all mutants, including those carrying a likely null allele, suggesting that it reflects the response to temperature of an independent factor which overlaps in function with *PHAN*. Null mutations that reveal the inherent temperature sensitivity of redundant pathways have previously been identified in *C. elegans* and *Drosophila* (e.g., Strome et al., 1995). The requirement for *PHAN* at the permissive temperature of 25°C appears minimal because all phan mutants closely resemble wild-type plants. A greater requirement, induced by growing mutant plants at the restrictive temperature of 15°C, reveals that *PHAN* is needed for all lateral organs to elaborate a proximodistal axis—primordia either fail to emerge or cease proximodistal growth at this temperature. In leaves, the requirement for *PHAN* extends from at least the stage immediately before appearance of primordia (P_0) into the stage after (P_1), because shifting mutant plants from 25°C to the restrictive temperature can prevent initiation from P_0 initials and growth of P_1 primordia, whereas older leaves remain unaffected. The role of *PHAN* function in primordium initiation and subsequent proximodistal growth coincides with its pattern of early expression as *PHAN* mRNA is confined to lateral organ initials and primordia. In leaves, it is first detected before primordia emergence in P_0 initials and persists in primordia until stages P_3–P_4.

PHAN Is Needed for Dorsoventral Asymmetry in a Subset of Lateral Organs

In addition to forming a proximodistal axis, lateral organs typically elaborate dorsoventral asymmetry. The temperature sensitivity of phan mutants allows the role of the *PHAN* function in these two processes to be partially separated. At the intermediate temperature of 17°C, the requirement for *PHAN* is reduced to a level sufficient for initiation of all organ primordia, although in leaves, bracts, and petal lobes it remains insufficient for determination of dorsal cell identity because these organs in phan mutants typically show no dorsoventral asymmetry and consist only of ventral cell types. Lack of dorsoventral asymmetry, including lateral growth, is consistent with the view that lateral growth results from *PHAN*-dependent dorsal cell identity. However, the alternative—that *PHAN* acts to specify lateral growth and that dorsal identity is a consequence of this—cannot be ruled out. Although primordium emergence occurs at 17°C, other aspects of proximodistal axis elaboration are not fully restored. Ventralized organs show reduced proximodistal growth and are therefore shorter than wild type, and they also exhibit reduced proximodistal asymmetry—for example, leaves show no distinction between distal blade and proximal petiole (Waites and Hudson, 1995). At 17°C, other floral organs—sepals, stamens, and carpels—develop normally, suggesting that other genes act redundantly with *PHAN* in these organs to specify dorsal identity and proximodistal growth after primordium initiation. Consistent with this, we have identified a *PHAN*-like gene that is expressed in inflorescences (unpublished data) and might therefore fulfill this role. At the higher temperature of 20°C, leaves, bracts, and petal lobes of *phan* mutants typically consist of mosaics of normal and ventralized tissues. Such mosaicism is consistent with dorsal and ventral identities being mutually exclusive and clonally heritable. Reduced dorsal specification in phan mutants could allow cells to assume ventral identity, rather than an intermediate state, and to give rise to a clone of ectopic ventral tissue.

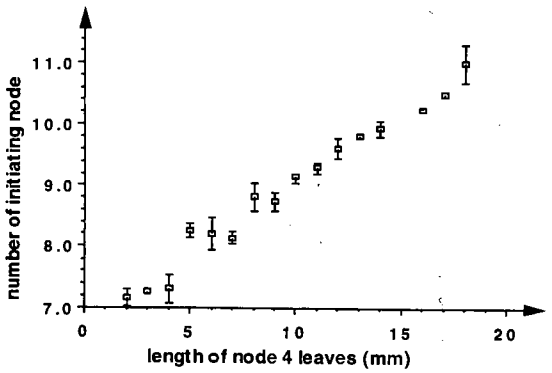

Figure 6. Estimating the Developmental Stage of the SAM from the Lengths of Existing Leaves

phan-607 mutants were grown at the permissive temperature of 25°C and the lengths of leaves at node 4 compared to the number of the node last initiated at the apex. The mean developmental stage of at least 15 plants at each leaf length was determined and plotted with standard errors.

Dorsoventral asymmetry only becomes apparent in *Antirrhinum* organs after they have begun to elaborate a proximodistal axis at primordium emergence. However, temperature shift experiments suggest that the requirement for *PHAN* function in dorsoventrality does not extend beyond the requirement in primordium initiation and outgrowth. Therefore, dorsoventrality may be specified within the shoot apical meristem (SAM), as previously suggested by surgical experiments in a number of species (Sussex, 1955; Snow and Snow, 1959; Hanawa, 1961). Determination of dorsoventrality in response to apical–basal polarity of the SAM would allow lateral organs to be oriented with reference to the stem axis.

Because *PHAN* is needed for the two key characteristics of lateral organs, its primary role may be to specify lateral organ identity as distinct from that of the meristem or stem. The site and timing of *PHAN* expression is also consistent with this role. Surgical experiments have suggested that the identity of leaves is irreversibly determined in P_0 initials (Snow and Snow, 1933), corresponding to the earliest stage of *PHAN* expression in *Antirrhinum*. Comparing the timing of *PHAN* with that of other genes, such as *FLO* and floral homeotic genes, which act as markers of lateral organ fate, suggests that it also has an early role in bract and floral organ development. That *PHAN* expression is limited to lateral organ initials implies that it interprets an existing prepattern. However, relatively little is known of the mechanisms that pattern the SAM and direct cells to lateral organ fates. Analysis of *Knotted1*-like homeobox genes, such as *STM* in *Arabidopsis*, has provided some insights. *STM* is expressed in SAM cells, and its down-regulation in P_0 initials provides an early marker of leaf fate (Long et al., 1996). Furthermore, loss of *STM* expression may be sufficient for lateral organ identity because the meristems of weak stm mutants can terminate at their summits in production of ectopic lateral organs (Clark et al., 1996). However, loss of *STM* activity may not be sufficient for determination of organ fate in normal positions, because ectopic expression of *STM*-like genes of the *Knotted1* family in the lateral organ initials of a number of species does not prevent primordium initiation (e.g., Smith et al., 1992; Matsuoka et al., 1993; Lincoln et al., 1994). In *Antirrhinum*, expression of the *STM* homolog disappears from leaf initials at about the time that they begin to express *PHAN*. Therefore, one explanation is that *STM* acts as a negative regulator of *PHAN* expression and organ identity, or vice versa.

Requirement for *PHAN* in Meristem Maintenance

Apical and axillary meristems of phan mutants that have ceased initiating leaves at the restrictive temperature do not continue to produce leafless stem axes. They lose their characteristic structure and show no evidence of continuing cell growth or division. However, apical cells can remain viable and not undergo irreversible differentiation, because they can regain meristematic activity on return to the permissive temperature. Because *PHAN* mRNA is restricted to lateral organ initials but is required for activity of the meristem as a whole, this function is likely to involve a *PHAN*-dependent signal originating from organ initials or primordia. Although several other genes have been identified that are necessary for meristem activity and elaboration of organ axes (Medford et al., 1992; Talbert et al., 1995; Laux et al., 1996; Pickett et al., 1996), none affect only lateral organ emergence and dorsoventrality but not activity of the meristem. Similarly, few higher plant species appear capable of producing stem axes without at least rudimentary lateral organs. Therefore, definition of lateral organ identity may not simply involve partitioning of meristematic cells into a more determined fate but may itself be necessary for activity of the stem cell population within the meristem.

Experimental Procedures

Plants and Growth Conditions

The origins of *phan* mutants and their wild-type progenitors have been described previously (Waites and Hudson, 1995). To analyze the effects of temperature, wild-type and *phan* mutant plants were grown from seeds that had been surface-sterilized to encourage germination and sown singly in 8 cm pots of Levington M3 compost. Because germination of both wild-type and *phan* mutants is inhibited at 15°C, newly sown seeds were maintained at 20°C for 7 days and subsequently transferred to growth rooms at 15°C or 25°C, with a 16 hr photoperiod provided by illumination from metal halide lamps (100 μmol $m^{-2}s^{-1}$ of photosynthetically active quanta). Germination was not apparent at the time of transfer. To estimate the developmental stage of plants at the time of temperature downshifts, a correlation was sought between the lengths of leaves that had emerged from the apical bud and the number of the node being initiated at the apex, determined by dissection. The morphology of the youngest leaf primordia (whether they overtopped the SAM or whether there was an obvious crease between their dorsal surface and the SAM) was used to divide further the interval between emergence of successive nodes. For both phan mutant and wild-type plants grown at 25°C, the lengths of leaves at node 4 provided a reliable indication of the developmental stage of initiating primordia at nodes 7–10 (Figure 6). Temperature shifts were performed in situ by heating or cooling over a period of 2 hr to avoid potential effects of differences in lighting between growth rooms.

Microscopy and In Situ Hybridization

Scanning electron microscopy was carried out as described previously (Waites and Hudson, 1995). Material for in situ hybridization was fixed, sectioned, and hybridized to digoxigenin-labeled RNA

probes as described by Bradley et al. (1993). Templates for transcription of *PHAN*-specific probes were derived from positions 705–1413 or 995–1413 of the *PHAN* cDNA, downstream of the conserved *MYB* region, subcloned in either sense or antisense orientations relative to the T7 promoter of pBluescript II (Stratagene). Templates for transcription of *FLO*, *DEF*, and *PLE* probes have been described by Coen et al. (1990), Sommer et al. (1990), and Bradley et al. (1993), respectively, and were kindly provided by these authors. The *Antirrhinum* homolog of the *STM* gene, used as an in situ hybridization marker, was obtained by probing an *Antirrhinum* inflorescence cDNA library with an *STM* cDNA clone at low stringency. The *Antirrhinum* gene, *AmSTM*, had the potential to encode a protein that was more similar to the *STM* gene product (73% identity to the 270 C-terminal amino acids of STM) than the product of *STM* was to that of the most closely related *Arabidopsis* sequence, *KNAT1* (54% amino acid identity in the same region). In common with all KNOTTED1-like proteins, AmSTM showed little sequence conservation in its N-terminal region. These results suggested that *AmSTM* was the likely ortholog of *STM*. Probes for in situ hybridization were transcribed from the 5′-UTR and the region of *AmSTM* encoding the poorly conserved 106 N-terminal amino acids.

Cloning *PHAN*

To identify a transposon responsible for the *phan*-552 mutation, the *phan*-552/*phan*-249G heterozygote was back-crossed to its *phan*-249G mutant parent to produce 13 families, each derived from a single seed capsule. From each family, DNA was extracted from a pool of PHAN$^+$ revertants and from a pool of mutant siblings and used in low-stringency Southern hybridization with a 600 bp probe derived from the 3′ end of *Tam4* (Luo et al., 1991). This allowed detection of a 6.3 kb BglII fragment that was present only in pools of mutant plants. High-stringency Southern hybridization with probes specific to individual members of the CACTA transposon family revealed that the fragment carried a copy of *Tam4*. A size-fraction of BglII-digested DNA containing only this copy of *Tam4* was restriction mapped by Southern hybridization, revealing that the transposon had inserted close to one of the BglII sites and that the shorter flanking region contained an EcoRI site in addition to that present in *Tam4*. To facilitate cloning, BglII fragments of ~6.3 kb were blunt-ended, ligated to EcoRI adaptors, and then digested with EcoRI. The left and right ends of *Tam4* were cloned into the EcoRI site of λgt10 from appropriate size fractions. Sequences flanking the right end of *Tam4* were subsequently used as probes in isolation of wild-type *PHAN* clones from cDNA and genomic libraries.

The start of *PHAN* transcription was mapped by 5′ RACE analysis using the modifications of Frohman and Martin (1989). cDNA was synthesized from mRNA expressed in inflorescence apices or vegetative shoot tips using a primer complementary to positions 1339–1362 of the cDNA. It was homopolymer-tailed and amplified sequentially using primers from positions 694–677 and 489–471 of *PHAN*.

Restriction mapping of the *phan*-249G allele revealed that it carried a DNA insertion within the coding region. An EcoRI fragment extending from within the insertion to a site beyond the 3′ end of *PHAN* was cloned from size-fractionated DNA. Once sequence analysis had revealed that the insertion was a copy of *Tam2*, primers complementary to the other end of *Tam2* were used to amplify a region that included the upstream junction. Other parts of the *PHAN* coding region were amplified from *phan*-249G and sequenced to confirm that the allele carried no further mutations. The *phan*-250G allele was found to contain a DNA insertion 3′ to an EcoRV site 2–8 bp upstream of the *PHAN* splice acceptor. PCR using a primer in the *PHAN* coding region (positions 694–677) was able to amplify DNA from both wild-type and *phan*-250G mutants when used with a second primer beginning at the *PHAN* initiation codon (positions 188–206), but it was able to amplify only wild-type DNA when used with a primer from the intron, suggesting that the insertion in *phan*-250G lay between the splice acceptor sequence and *PHAN* protein coding region. The restriction map of the insertion did not correspond to that of any known transposon.

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EMBL Database Accession Number

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